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Manuscripts and editorial correspondence should be addressed to

Acta Agronomica Hungarica
Agricultural Research Institute of the
Hungarian Academy of Sciences
H-2462 Martonvásár, Hungary
Phone: (36-22) 460-016
Fax: (36-22) 460-213

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HEAT STRESS-INDUCED DIFFERENTIAL ALTERATIONS IN THE PHOTOSYNTHESIS, MEMBRANE THERMOSTABILITY AND BIOMASS PRODUCTION OF BREAD AND DURUM WHEAT VARIETIES

G. GALIBA[1], Z. NAGY[2], T. JANDA[1], K. SZENTE[2], Z. CSINTALAN[2], R. TUBEROSA[3], P. MONNEVEUX[4] and M. COUMANS[5]

[1]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, MARTONVÁSÁR, H–2462 HUNGARY,
[2]PLANT PHYSIOLOGICAL SECTION, DEPARTMENT OF BOTANY AND PLANT PHYSIOLOGY, AGRICULTURAL UNIVERSITY OF GÖDÖLLŐ, H–2103 GÖDÖLLŐ, HUNGARY
[3]DIPARTIMENTO DI AGRONOMIA GENERALE, FACOLTÁ DI AGRARIA, UNIVERSITÁ DI BOLOGNA, VIA FILIPPO RE 6–8, I–40126 BOLOGNA, ITALY
[4]CHAIRE DE PHYTOTECHNIE, STATION D'AMÉLIORATION DES PLANTES ENSA-INRA, PLACE VIALA, 34060 MONTPELLIER CEDEX 01, FRANCE
[5]UNIVERSITÉ MONTPELLIER II, SCIENCES ET TECHNIQUES DU LANGUEDOC, PLACE EUGÉNE BATAILLON, 34095 MONTPELLIER CEDEX 5, FRANCE

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Ten hexaploid bread wheat (*Triticum aestivum* L., genomic composition AABBDD) and ten durum wheat (*Triticum turgidum* L., var. *durum;* genomic composition AABB) genotypes were compared for heat tolerance following treatment for one week at 32/27°C and one week at 37/27°C D/N in growth chambers. The control plants were raised under an 18/15°C D/N temperature regime. As a consequence of the high temperatures, the biomass production of durum genotypes was more severely affected than that of bread wheats, although the cell membrane thermostability of durum genotypes was higher. Net photosynthesis (A) declined considerably in both species, reaching a negative carbon balance in durum genotypes. No substantial decrease was observed in the fast component of chlorophyll fluorescence, indicating that the thylakoid membranes remained intact. The decrease in the slow quenching of induced chlorophyll fluorescence indicated that the plants experienced a superoptimal temperature for the C fixing pathway enzymes during the heat treatment. The crown region of durum genotypes suffered significantly more water loss than bread wheats (16% and 8%, respectively). The abscisic acid (ABA) concentration reached significantly higher values in durum wheats. The increased temperature of the leaf tissue coupled with a high level of ABA accumulation accelerated the processes of senescence in the case of durum genotypes, causing an increased photorespiration rate and a negative carbon balance.

Key words: *Triticum aestivum* L., *Triticum turgidum* L. var. *durum,* abscisic acid, heat tolerance, membrane thermostability, photosynthesis, water stress, wheat

Introduction

All plant processes are sensitive to and can be irreversibly damaged by heat if plants are exposed to sufficiently high temperatures for an adequate length of time. The productivity of wheat and other temperate zone species falls markedly at high temperatures (Blum, 1988). The optimum temperature for photosynthesis and reproductive growth is near 20°C for wheat (Al-Khatib and Paulsen, 1984). The net uptake of carbon dioxide decreases at the high daytime temperatures commonly experienced in natural environments (Berry and Björkman, 1980). Temperatures commonly exceed 35°C before physiological maturity in many wheat-growing regions and therefore constitute an important constraint to productivity. The increase in respiration rate and the excessive consumption of photosynthates due to the increased temperature may injure the protoplasm. Such chronic effects of high temperature stress usually result in suppressed growth and reduced yield, but rarely cause lethal injury to the plants (Christiansen, 1978; McWilliam, 1980).

High temperature alters the efficiency of photosynthesis and the relative amount of light energy that is re-emitted as chlorophyll fluorescence (Krause and Santarius, 1975; Schreiber and Berry, 1977; Weis, 1982). The amount of chlorophyll fluorescence indicates thylakoid membrane integrity and the relative efficiency of the electron transport from photosystem II (PSII) to photosystem I (PSI) (Krause and Weis, 1984). For example, when leaves are submitted to heat stress conditions, there is a marked increase in the dark level of *in vivo* chlorophyll fluorescence (F_O) (Schreiber and Berry, 1977). This effect presumably reflects the physical dissociation of the PSII reaction centres from the light harvesting system (Armond et al., 1979). The variable fluorescence level (Fv) (difference between maximal fluorescence level (Fm) and Fo) has been proposed as an especially useful measure of heat-induced injury in wheat (Moffatt et al., 1990). The temperature at which F_O started to rise was in positive correlation with the heat tolerance of durum wheat cultivars (Havaux et al., 1988). The apparent problem of this kind of evaluation is that although the water-splitting component of PSII may be particularly sensitive to heat (Berry and Björkman, 1980), the threshold temperatures required to cause irreversible damage were quite high (more than 40°C for a species adapted to cool seasons).

In contrast to heat stress, water stress does not significantly modify the F_o level (Havaux et al., 1986). Leaf dehydration appears to primarily affect other fluorescence parameters, namely the slow quenching of induced chlorophyll fluorescence (Havaux and Lannoye, 1985a, b; Lichtenthaler et al., 1986; Ali Dib et al., 1994). In this phase, apart from photochemical quenching, non-photochemical quenching mechanisms play a substantial role (Krause and Weis, 1991).

A cell-membrane system that remains functional during heat stress appears central to the adaptation of plants to high temperature (Raison et al., 1980). Sullivan and Ross (1979) used a test that measures the amount of electrolyte leakage from leaf discs bathed in deionized water after exposure to heat-shock treatment. They interpreted the measurement as an indicator of cell membrane thermostability (MT) in response to heat stress. They used this procedure to identify genetic variations in heat tolerance in sorghum (*Sorghum bicolor* L.). The variation observed in MT was related to differences in whole plant photosynthesis and field performance under high temperature stress. Since then, several laboratories have used this method for predicting the heat tolerance of different plant species (reviewed by Blum, 1988 and Hall, 1992). The result of the test depends greatly on the environmental conditions to which the plants are exposed prior to sampling and it was also noted that plant tolerance to heat stress may change with age (Blum and Ebercon, 1981; Shanana et al., 1990). Chen et al. (1982) reported that genetic differences in MT are also dependent on the hardening temperature prior to heat stress.

The endogenous abscisic acid (ABA) concentration in plants increases following heat treatment (Blum, 1988). Exogenously applied ABA induced heat tolerance in cultured cells of grape (Abass and Rajashekar, 1993) and *Bromus inermis* Leyss (Robertson et al., 1994). Increased heat tolerance was achieved by *in vitro* selection for ABA insensitivity in wheat (Lu et al., 1989). High temperature stress did not affect the chloroplast structure in a maize line exhibiting a high ABA concentration but damaged many chloroplasts of another line containing low ABA (Ristic and Cass, 1992). These results suggest that ABA may be a factor in high-temperature acclimatization and heat tolerance induction.

The work described here compares the effects of elevated temperatures on the biomass production, ABA concentration, photosynthetic characteristics and membrane thermostability of several bread wheat and durum wheat genotypes in the seedling stage. The main objectives of this study were 1) to determine whether there was any advantage in the presence of the D genome in hexaploid bread wheat genotypes (genomic composition AABBDD) over tetraploid durum wheat (genomic composition AABB) during adaptation to elevated temperatures, and 2) to elucidate whether the investigated traits play a substantial role in the adaptation processes of wheat grown under a 37/27°C (day/night) temperature regime.

Materials and methods

Plant material and growth conditions

The experiments were carried out in the phytotron at the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár. A complete list of the bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L., var. *durum*) genotypes (with their geographic origin) examined in the present work is given in Table 1. The genotypes were selected from different geographic regions to represent the inherent genetic diversity of cultivated bread and durum wheats.

Seeds were grown in a 4:1 mixture of heat-sterilised garden soil and sand in pots (12 × 9 × 18 cm) and placed in two PGV-36 Conviron chambers. The day/night (D/N) temperatures in the first and second weeks were 15/10°C and 18/15°C, respectively, with a 16 h photoperiod and 300 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density. The relative humidity of the ambient air was 70/75%. In the control chamber the 18/15°C values were maintained for two additional weeks.

Table 1
Bread and durum wheat genotypes used in heat tolerance experiments

Genotypes	Origin
Bread wheat	
1. Kalyansona	(India)
2. Wichita	(USA)
3. ND 7532	(USA)
4. Kharchia	(India)
5. Maris Hobbit	(Great Britain)
6. Cappelle Desprez	(France)
7. Chinese Spring	(China)
8. Cheyenne	(USA)
9. Regina	(Germany)
10. Hope	(Great Britain)
Durum wheat	
11. Simeto	(Italy)
12. Adamello	(Italy)
13. Ofanto	(Italy)
14. F22T	(Italy)
15. DF–104–85	(Romania)
16. GK Minaret	(Hungary)
17. Korall	(Ukraine)
18. Korund	(Russia)
19. Windur	(Germany)
20. 720	(Bulgaria)

In the heat treatment the plants were exposed to 32/27°C and 37/27°C in the third and fourth weeks, respectively. To avoid heat-induced water deprivation the pots in the heat treatment were watered daily. Three plants were grown in each pot. The design was a randomized complete block, with four replications. The data were analysed by the STATGRAPHICS statistical package, using t-test and ANOVA function to assess significant differences between means.

Biomass and water content measurements

The shoot biomass of the seedlings was sampled after 2 weeks of growth at 32/27°C and 37/27°C D/N, dried at 70°C for 48 h, and weighed. The mean values of 12 replicates are presented as a percentage of the control (grown at 18/15°C). For each variety, the deviation from the grand mean of the heat treatment is shown. The average water content of the shoots (crown + whorl region) and leaves was calculated separately from the fresh and dry weights of these samples. The deviation of the mean values (calculated from 12 replicates) from the control can be seen in the figures.

Abscisic acid determination

Leaf samples were taken from six replicate plants after 2 weeks of growth at 32/27°C and 37/27°C D/N for ABA determination by radioimmunoassay (RIA) using the monoclonal antibody MAC62 (synonym MAC252) described by Quarrie et al. (1988). The samples were freeze-dried, powdered, and shaken in water (1 : 10; weight : volume) overnight at 4°C in the dark to extract ABA. The assay was performed on duplicate 50 μl aliquots, essentially as described by Pekic and Quarrie (1987). The assay was validated for crude aqueous extracts of wheat leaves by Quarrie et al. (1988).

Membrane thermostability

The seeds were germinated on wet germination paper and maintained at 15°C under dark conditions. The seedlings were evaluated for membrane thermostability (MT) using the procedures described by Saadala et al. (1990). This included germinating the seedlings for 10 to 14 days, hardening the seedlings for 48 h at 34°C in a thermostat, sampling plant material and evaluating MT. Leaf segments (2 cm long) from 10 seedlings per genotype were exposed to 49 and 52°C for one hour. Membrane thermostability was expressed as percentage relative injury: $RI = 100T_1/T_2$. Briefly, RI was determined by measuring the amount of electrolyte leakage (T_1) from plant tissue (ASA 610 Automatic Seed Analyser, U.S.A) in response to heat treatment (49 and 52°C) relative to the total amount of electrolyte (T_2) released from the same plant tissue upon autoclaving, which is presumably associated with MT (Tahir and Singh, 1993). Values for RI range between 0 and 100%, with low values indicating limited membrane injury and high values greater membrane injury.

Measurement of net CO_2 gas exchange rates and stomatal conductance in leaves

Net CO_2 gas exchange rates were measured using an LCA2 type infrared gas analyser equipped with a PLC-N leaf chamber (Protection Engineering Ltd., Hoddesdon, UK) on five replicate plants of bread (cvs. Kalyansona, Wichita, Maris Hobbit, Cappelle Desprez, Chinese Spring and Cheyenne) and durum (cvs. Simeto, F22T, Korund and Windur) wheat after one week of exposure to 32/27°C D/N temperatures. In the following week, the plants were exposed to 37/27°C and the leaf CO_2 gas exchange rates were measured again. Gas exchange rates were calculated according to von Caemmerer and Farquhar (1981).

Adaxial and abaxial stomatal conductances were measured at the end of the 37/27°C D/N temperature treatment in parallel with the CO_2 gas exchange on the same leaves using an AP4 porometer (Delta T Dev. Ltd.). Total (adaxial+abaxial) conductances are reported.

Chlorophyll fluorescence measurements

The chlorophyll fluorescence induction parameters of the leaves of all the varieties were determined at room temperature using a pulse amplitude modulated (PAM) fluorometer (PAM–2000, Walz, Effeltrich, Germany). The saturating photosynthetic photon flux density was 2500 $\mu mol\ m^{-2}s^{-1}$. Before the measurements, the plants were dark-adapted for 30 min. For the chlorophyll fluorescence induction parameters the nomenclature of van Kooten and Snel (1990) was used. The results are the means of 5 replications. The t-test was used for statistical analysis. The slow kinetics of fluorescence induction was measured with a He-Ne laser fluorimeter connected to a computer. The fluorescence decrease ratio (Rfd = M/(M-S), where M: fluorescence maximum level, S: steady state fluorescence level) described by Lichtenthaler et al. (1986) was used as the test parameter.

The measurements of net photosynthesis, stomatal conductance and slow induction kinetics of fluorescence decay were carried out in parallel; therefore, only a limited number of the genotypes could be included.

Results

Biomass and water content

Heat treatment considerably decreased the biomass production in all the genotypes examined. The durum genotypes, with one exception, were more retarded than the bread wheat genotypes (Fig. 1). The average biomass production of the durum genotypes reached 45.9% of the control, while this value was significantly higher ($LSD_{0.001}$ = 10.6) for bread wheat varieties (67.6%). The water content in the leaves of both durum and bread wheat genotypes decreased in the same manner slightly, but significantly ($LSD_{0.05}$ = 4.79) from the 85.9% control value to 80% after the two-week heat treatment. However, in the crown samples of durum varieties (crown + whorl region together) the decrease in water content was significantly higher than in the crowns of bread wheat varieties (Fig. 2). The water content was originally the same in both species (89.2%), while it decreased to 73.3% in durum and to 81.8% in bread wheat genotypes ($LSD_{0.05}$ = 6.5) following the 2-week treatment.

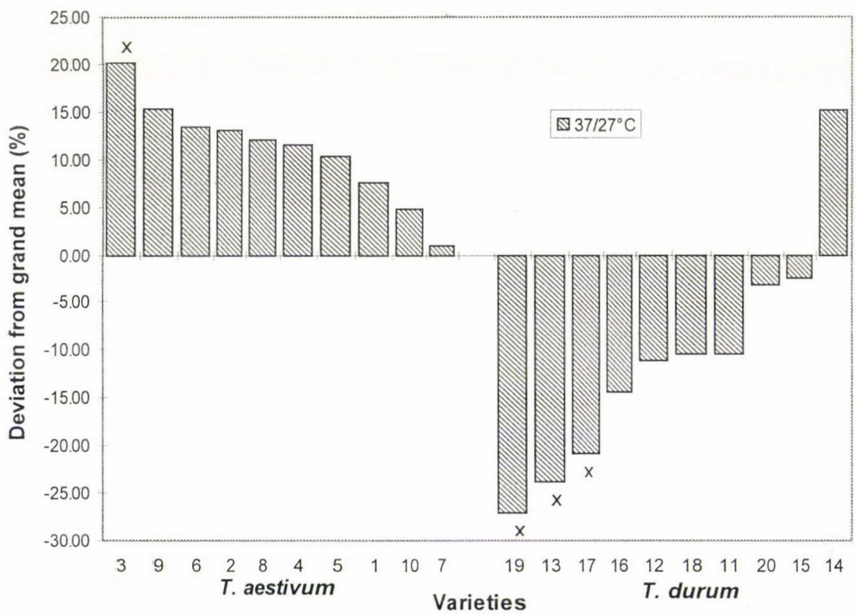

Fig. 1. The effect of two-week heat treatment, applied as one week at 32/27°C and another week at 37/27°C (D/N) consecutively, on the biomass of 10 bread and 10 durum genotypes. The means of 12 replicates are presented on a dry weight basis as a percentage of the control. The deviation of these means from the grand mean of the two-week heat treatment is shown for each genotype. The genotypes corresponding to each number are listed in Table 1. The bread wheats were ranked in descending order and the durum ones in ascending order. Significant differences from the corresponding treatment grand mean are shown by (x), (xx) and (xxx) at levels of p<0.05, 0.01 and 0.001, respectively

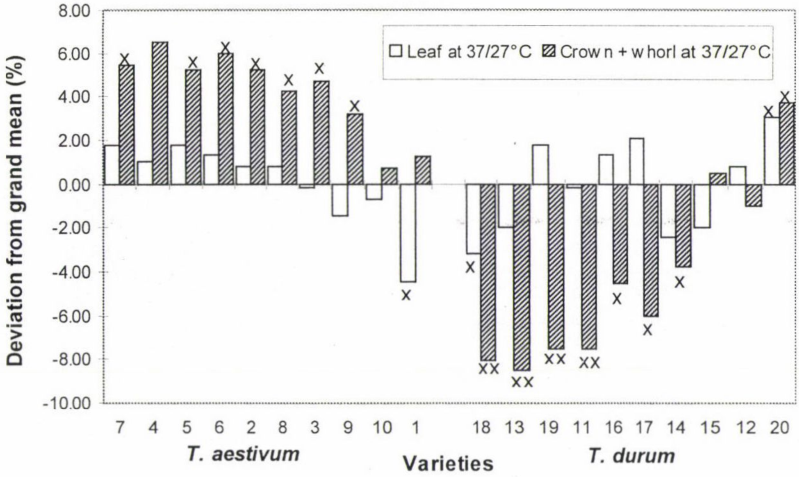

Fig. 2. The effect of two-week heat treatment, applied as one week 32/27°C and another week at 37/27°C (D/N) consecutively, on the water content (%) in the leaves and whorl parts of 10 bread and 10 durum wheat genotypes.
The means of 12 replicates were calculated. The deviation of these means from the grand mean of the two-week heat treatment is shown for each genotype. Symbols and abbreviations as in Fig. 1.

Abscisic acid

The mean ABA concentration in the leaves of the bread and durum wheat genotypes grown under control conditions was similar, being 352.9 ng/g D.W. ($LSD_{0.05}$ = 74) and 366.9 ng/g D.W. ($LSD_{0.05}$ = 81), respectively. Following the 2-week heat treatment, the ABA concentration increased considerably, up to 633 ng/g D.W. ($LSD_{0.05}$ = 104) on average in durum genotypes. The ABA level remained significantly lower at 492.8 ng /g D.W. ($LSD_{0.05}$ = 106) in bread wheat varieties In the nine durum genotypes (because of permanent yellowing and wilting, the leaves of Ofanto were not suitable for ABA extraction) the increment was significant in five cases ($p < 0.05$), while in the ten bread wheat varieties only one showed a significant increase (Fig. 3).

Membrane thermostability

The RI values determined by the MT test were highly dependent on the testing temperatures. Treatment at 49°C for 1 h was not high enough to discriminate among the genotypes (Fig. 4). Applying 52°C for 1 h discriminated well between the durum and bread wheat genotypes. The average value of RI reached 71.1% for bread wheat, while it was only 41.9% ($LSD_{0.001}$ = 14.0) for durum genotypes.

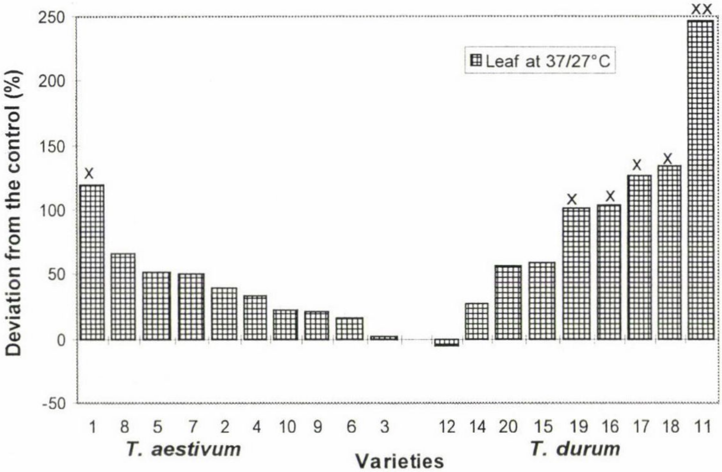

Fig. 3. The effect of two week heat treatment, applied as one week at 32/27°C and another week at 37/27°C (D/N) consecutively, on the abscisic acid content in the leaves of 10 bread and 9 durum wheat genotypes.
The means of 12 replicates are presented as a percentage of the control. The deviation of these means from the mean of the control is shown for each variety. Symbols and abbreviations as in Fig. 1.

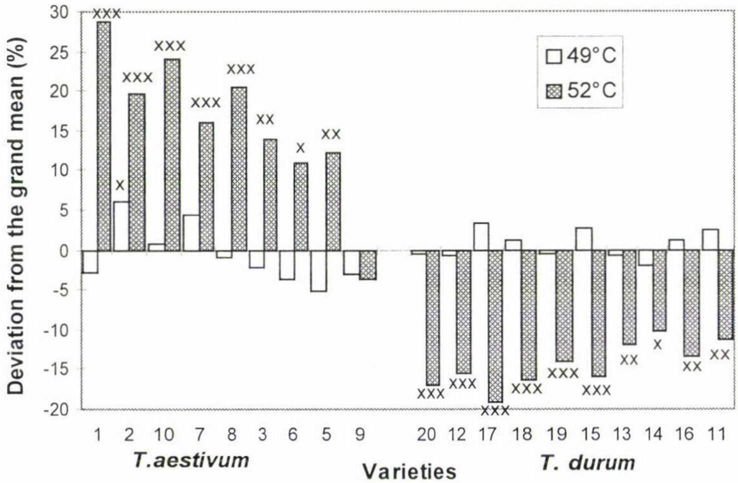

Fig. 4. The effect of heat treatment on the membrane thermostability of 10 bread and 10 durum wheat genotypes.
Leaf segments (2 cm long) of 10 hardened seedlings per genotype were treated at 49 and 52°C for one hour. The hardening of the seedlings was carried out in a thermostat at 34°C for 48 h. Membrane thermostability was expressed as percentage relative injury (RI). The means of 10 replicates were calculated. The deviation of these means from the grand means of the two heat treatments is shown for each genotype. Symbols and abbreviations as in Fig. 1

Chlorophyll fluorescence measurements

The F_v/F_m ratio indicates the potential yield of the photochemical reaction of PSII, and a decrease in this parameter is the most reliable sign of photoinhibition (Krause, 1988). This parameter hardly changed even after 1 week at 37°C (max. 7% difference between the treated and control plants). Very slight but significant decreases were observed in the case of ND7532, Maris Hobbit, Chinese Spring, Capelle Desprez and Regina, among the ten *T. aestivum* varieties, but there were no significant changes in the case of durum genotypes (data not shown). These results suggest that after a 1-week heat stress carried out at 32°C and 1 week at 37°C the effect of photoinhibition is negligible.

The fluorescence decrease ratio (Rfd) was greatly reduced in both the bread and durum wheat plants following the 1-week 32/27°C treatment, reaching values around 50% of those in the control plants (Table 2). The lowest values were those of the durum lines Korund and Windur.

After the second week of heat treatment (i.e. 37/27°C) Rfd decreased most markedly in the bread wheat cultivar Maris Hobbit, which also showed a decrease in the fast component Fv/Fm (see above). The treated/control Rfd ratios further decreased (Table 2) in all genotypes except for Chinese Spring (bread) and Windur (durum). Since the fast component Fv/Fm did not show any considerable change due to the heat treatment, the decrease in Rfd suggests that inhibition in the dark phase of CO_2 fixation is of greater importance. This latter might be the result of both the direct effect of superoptimal temperatures on the enzymes of the C_3 carbon fixation pathway and the indirect effect of dehydration.

Net CO_2 gas exchange rate and stomatal conductance

The net CO_2 uptake of each genotype decreased to 70–85% of the control at the end of the 32°C treatment (Table 3). In the second week (37°C) the bread wheat genotypes showed values around 0, while the durum wheats had a strongly negative (instantaneous) carbon balance together with visible symptoms of senescence (e.g. permanent wilting and yellowing of the leaves). It should be noted that all the durum genotypes had slightly higher treated/control Rfd ratios than the bread wheats (Table 2) in spite of the strong respiration activity in the light.

Stomatal conductance also decreased in all genotypes (Table 3), most markedly in the durum wheats Simeto and F22T. At the same time, Simeto and F22T showed the highest ABA concentrations (Fig. 3) and the most advanced stage of senescence.

Table 2

Effect of two-week heat treatment, applied as one week at 32/27°C day/night (D/N) and another week at 37/27°C, consecutively on the fluorescence decrease ratio (Rfd) values of 6 bread and 4 durum genotypes. The treated control ratios of Rfd for the first and second weeks are also presented. Control plants were grown at 18/15°C (D/N). Each value represents the mean of 5 replications

Genotypes	Fluorescence decrease ratio (Rfd)				$Rfd_{treated}/Rfd_{control}$	
	First week		Second week		First week	Second week
	Control	Treated	Control	Treated		
Bread wheat						
1	2.9	1.4	3.0	1.4	0.48	0.49
2	2.6	1.6	2.9	0.9	0.60	0.26
5	2.6	1.5	3.3	0.7	0.58	0.20
6	3.1	1.7	3.5	1.2	0.46	0.26
7	3.3	1.4	2.8	1.3	0.41	0.44
8	2.5	1.7	2.3	0.8	0.63	0.27
Durum wheat						
11	2.2	1.8	1.9	0.8	0.82	0.41
14	3.0	1.7	2.7	1.2	0.58	0.43
18	1.9	1.1	1.3	0.6	0.60	0.42
19	2.9	1.2	1.4	0.7	0.41	0.42
$LSD_{(P=0.05)}$	0.6	0.4	0.8	0.4		

Discussion

As expected, high temperature stress adversely affected the biomass production of bread and durum wheat genotypes. The rate of decline was more marked in durum wheat genotypes for this trait (Fig. 1). Al-Khatib and Paulsen (1984) applied temperature regimes similar to our experimental conditions to study the mode of high temperature injury to bread wheat. They concluded that a major effect of high temperature is the acceleration of senescence, including the cessation of vegetative growth, the deterioration of photosynthetic activities and the degradation of proteinaceous constituents. Our results support their findings and indicate that the symptoms of senescence are more pronounced in durum genotypes than in bread wheats.

Although the relationship between fluorescence and the electron transport is rather empirical, there are many indications that it remains valid under a variety of circumstances (Cornic and Britains, 1991; Oberhuber et al., 1993). Simultaneous measurements on net photosynthetic rate (A) and fast fluorescence kinetics were taken on the flag leaves of field-grown bread and durum wheat from anthesis to senescence (Di Marco et al., 1994). The electron transport rate (calculated from the fluorescence) declined less than A during leaf senescence. The increasing discrepancy between A and the electron transport

Table 3

Effect of two-week heat treatment, applied as one week at 32/27°C, day/night (D/N), and another week at 37/27°C consecutively on the net CO_2 uptake and on the total (abaxial + adaxial) conductances of 6 bread and 4 durum genotypes. Control plants were grown at 18/15°C (D/N). Each value represents the mean of 5 replications.

Genotypes	CO_2 uptake (μmol CO_2 m^{-2} s^{-1})				Conductance (mmol H_2O m^{-2} s^{-1})	
	First week		Second week		Second week	
	Control	Treated	Control	Treated	Control	Treated
Bread wheat						
1	6.2	4.0	5.8	–0.5	218	138
2	4.9	3.6	4.5	–0.3	311	161
5	4.3	3.8	4.4	0.2	244	117
6	5.3	3.5	4.7	–1.2	268	183
7	5.2	3.7	4.0	0.2	156	203
8	5.8	4.0	3.7	–0.5	310	147
Durum wheat						
11	5.8	4.6	5.9	–7.2	314	73
14	5.6	4.5	4.5	–10.3	375	247
18	4.9	3.4	4.2	–34.5	408	28
19	5.0	3.4	2.1	–25.3	284	263
$LSD_{(P=0.05)}$	1.3	0.5	1.1	1.2	48	34

during leaf senescence could partly be explained by an increment in the photorespiration rate (Di Marco et al., 1994). This phenomenon may also have occurred under the present experimental conditions (Table 3). Most likely, a dramatic increase in respiration caused a negative carbon balance in all the durum genotypes following the second week of heat treatment. Seemingly, the bread wheats were still able to maintain an equilibrium between the anabolic and catabolic processes.

Fluorescence parameters clearly show that the thylakoid membranes of the durum varieties remained intact during heat treatment. This probably indicates that the electron transport remained constant. The slight decrease in the F_v/F_m ratio in the case of five of the ten bread wheat genotypes indicates that some damage occurred in their thylakoid membranes, although this slight alteration did not really affect their performance. As was pointed out in the introduction, the changes in F_0 values indicate the degree of damage to the thylakoid membrane. The critical temperatures at which F_o starts to increase have been shown to be above 45°C, while the temperatures of maximal fluorescence ranged between 54 and 63°C in different durum wheat, barley and *Triticale* varieties (Havaux et al., 1988). Consequently, the severe retarding effect of heat in the temperature regime of 27–37°C used in our experiment originated from some alteration other than the heat-induced structural disorganisation of the thylakoids.

The parameter Rfd (computed from the slow component of fluorescence kinetics) proved to be a good indicator under the present heat stress conditions (Table 2). The substantial drop in the fluorescence decrease ratio indicated the role of non-photochemical quenching mechanisms (Krause and Weis, 1991). Havaux and Lannoye (1985b) observed that leaf dehydration induces a substantial inhibition of the slow quenching of induced fluorescence in leaves submitted to a dark/light transition (Kautsky effect). This effect appeared, however, to be much more pronounced in drought-sensitive varieties than in resistant ones, suggesting that the retention of fluorescence quenching activity may be a good indicator of drought resistance (Ali Dib et al., 1994). In our particular case, despite daily watering, the water content of the plants decreased slightly as a secondary effect of heat treatment. The 5% decrease in the water content of the leaves does not interfere with the photosynthetic electron transport, which has been shown to be very resistant to dehydration (Kaiser, 1987; Cornic and Britains, 1991). The consequence of the substantial decrease in the water content of the crown region proved to be much more important during heat stress. The average decrease in the water content of the crown regions was significantly ($p < 0.05$) higher in durum than in bread wheat, which was the likely cause of the higher ABA concentration in the leaves of durum genotypes (Figs. 2 and 3). A high degree of water loss in the crown region was associated with high ABA concentrations and the largest decrease in stomatal conductance (Table 3). The ABA concentration increased significantly in the leaves of one bread wheat variety (Kalyansona), the only one suffering from a significant water loss. Among the durum genotypes, Adamello, F22T, DF–104–25 and 720 had the highest water contents and the lowest endogenous ABA levels. The biomass production of these varieties were also among the least affected by the enhanced temperatures (Fig. 1).

The better performance of bread wheat varieties compared to durum genotypes at the seedling stage might be due to their superior water retention capacity under our particular experimental conditions. The importance of the superior osmoregulation capacity of bread wheat varieties over durum wheats under dry conditions has also been reported (Morgan et al., 1986; Gavuzzi et al., 1993). Havaux et al. (1988) found that the most heat-tolerant cereal varieties were also the most drought-tolerant ones.

The lower RI values clearly show the superior membrane thermostability of durum over bread wheat varieties (Fig. 4). When evaluating the heat tolerance by MT measurements in wild relatives and primitive forms of wheat, Damania and Tahir (1993) found durum wheat varieties to be, on average, the most tolerant to heat. Bread wheat showed only moderate tolerance. *Triticum dicoccoides,* the wild progenitor of durum wheat, was surprisingly the most susceptible to heat. At the same time, highly tolerant lines of *Aegilops tauschii* (the donor species of the D genome) were identified. The maximum temperature used in the present experiment was 37°C, while 49°C was still not high enough to discriminate among the durum and bread wheat varieties (Fig. 4). Consequently, membrane thermostability did not significantly affect the level of heat tolerance under our experimental conditions. The importance of the relative stability of the carbon exchange rate over membrane thermostability as a selection index in wheat for improved production in hot, dry environments was also emphasized by Blum (1986).

Our results suggest that hexaploid wheat may have some advantage over tetraploid forms at elevated temperatures. The better performance can be attributed at least partly to the better water-saving capacity. The increased leaf tissue temperatures coupled with water depletion seem to accelerate the process of senescence. The concomitant increase in ABA concentration in the leaves may also be a sign of the severity of the stress impact, but it is part of the adaptive response of the plant to drought. To elucidate this particular point further research is required.

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THE NUTRITIVE QUALITY OF LEGUME FOODSTUFFS PRODUCED UNDER DRY GROWING CONDITIONS

E. NEMESKÉRI

UNIVERSITY OF AGRICULTURAL SCIENCES, DEBRECEN, HUNGARY

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The nutritive quality and trypsin inhibitor activity were investigated in early, mid-early and late maturing soybean cultivars, in white and coloured dry beans, and in dry pea cultivars, grown under irrigated and non-irrigated conditions. The enzyme inhibition was too low to be measured in dry peas. The trypsin inhibitor activity, independent of growing conditions and ripening time, was greater in soybeans than in beans. Investigations into the effect of drought demonstrated that trypsin inhibitor activity (TIA) significantly increased in both soybeans and beans grown without irrigation, except in white bean lines selected for low TIA. The nutritive quality of both species was improved by irrigation. The oil accumulation decreased in the seed of mid-early soybean cultivars grown under dry conditions.

Key words: nutritive quality, trypsin inhibitor, legume foodstuffs, drought

Introduction

Edible legumes are important sources of vitamins and energy in human alimentation. Because of their positive dietary effects, the consumption of legumes is highly recommendable for diabetics and for individuals with high blood cholesterol levels (Antal and Bíró, 1991).

Their seeds are rich sources of fibre, but the availability of this fibre is inhibited by their phytate contents. These phytates form complexes with various minerals, such as zinc, calcium, iron and copper, and thus decrease fibre availability (Antal, 1993; Walker, 1981). In different age-groups, for example in children, the proportion of food legumes in the diet is limited by chemical components in the grain, as well as by the amount of antinutritive factors (Brandon et al., 1991). Although the antinutritive material found in soybeans is decomposed by subjecting it to heating, nevertheless, foodstuffs with low levels of Kunitz trypsin inhibitors and Bowman-Birk trypsin-chimotrypsin inhibitors contain significant amounts of specific inhibitors, even after heat processing.

Animal feed tests proved that alkali treatment caused changes in the structure of amino acids in bean flour, resulting in the production of other, toxic compounds, such as lysinoalanine (Wyckoff et al., 1983; Williams et al., 1984; Pusztai et al., 1979). The low digestibility of dry bean protein has been attributed to the proteolysis-resistant, large, storage protein (phaseolin) fibre, tannins and other polyphenols (Philips et al., 1983; Donatucci, 1983). Other studies have pointed out the role of lectins in contributing to the low

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digestibility of bean proteins, rather than that of trypsin inhibitors (Van de Poel, 1990).

A high positive correlation has been found between coloured seed coat, antitrypsin activity and tannin contents (Elias et al., 1979). Some soybean cultivars were found to produce good quality flour after a brief subjection to heating to deactivate the trypsin inhibitors (Friedman et al., 1991). However, during the heat processing, both the lysine content and the available lysine in the soybean protein decreased (Mao et al., 1993).

Grain quality is dependent on cultivars and growing conditions (Kochhar et al., 1988; Gatehouse et al., 1979). Significantly lower trypsin inhibitor activity (TIA) has been found in cowpeas than in other legumes (Bressani and Elias, 1977).

An increase in protein does not necessarily mean that there will be any quality improvement. In the soybean lines selected for high protein content, only the concentrations of the amino acids arginine and glutamine were higher in some lines than in the standard (Serretti, 1994).

In this study, we investigated how the grain quality and trypsin inhibitor activity were changed under dry growing conditions in soybean, bean and pea cultivars.

Materials and methods

From 1992 to 1994, investigations were made on eleven white and eleven coloured dry bean lines, three early, three late and two mid-early soybean cultivars, and on dry peas.

The cultivars and breeding lines were planted in field experiments with four random replications on chernozem soil. Although drought conditions prevailed in 1992 and 1993 during the generative periods of the plants, we did not irrigate, in order to test the effect of drought on grain quality and trypsin inhibitor activity (TIA). In 1994, the climatic conditions were the same as previously, but this time we irrigated the plants during flowering.

After harvesting, we gathered 100 g seed samples from the four replications, and analysed them in the Central Laboratory of the Agricultural University, Debrecen. The determinations of trypsin inhibitor activity were conducted according to the MSZ-081833-83 Hungarian standard, which corresponded to the method of Smith et al. (1990). The degree of enzyme inhibition was expressed in trypsin inhibition units (TIU) per mg fat-free dry matter. The measurement of seed protein was made with the Kjel-Foss method and expressed in terms of dry matter. The fat content was determined using the Soxhlet extraction method. The results have been appraised using ANOVA statistical methods.

Results and discussion

The results showed that the protein content, fat content and trypsin inhibitor activity were greater in soybeans than in other beans (see Tables 1 and 2). Although the seed protein content of the early group of soybeans was similar to that of the mid-early ones, approximately 33%, TIU proved to be the largest in the latter group (Table 1). The late maturing soybean cultivars were characterised by high protein content and relatively low TIU.

Table 1

Some growing properties and nutritive quality of soybean seeds in different cultivars. [Trypsin inhibitor amounts are expressed in trypsin inhibition units (TIU) per mg fat-free dry matter; fat and protein contents are expressed as a percentage of dry matter] 1992–1994

Cultivar	Days to maturity	Thousand grain weight (g)	TIU	Fat (%)	Protein (%)
Early maturing					
Mc Call	113	131	38.50	19.67	34.42
BS-38	113	171	40.35	19.74	32.79
HM-262	125	154	33.35	20.21	34.74
Mid-early					
Evans	132	157	40.44	19.29	33.33
Eszter	132	139	42.01	17.76	33.63
Late maturing					
K 5846	148	127	28.74	18.77	36.44
Panther	147	172	32.15	16.40	38.91
Borza	166	159	28.58	17.80	34.35

In dry peas, enzyme inhibition could not be measured, contrary to findings in the studies of Vetter et al. (1984), who showed differences in TIA among the varieties.

White seeded bean lines selected for low trypsin inhibitor activity and non-selected coloured seeded lines were planted under dry growing conditions, from 1992 to 1994. As a result of selection, trypsin inhibitor activity decreased in white seeded bean lines, when compared with coloured seeded lines, together with a slight increase in protein content (Table 2).

Our results concerning the TIA of beans with various seed coat colours are inconsistent with those of Wyckoff et al. (1983), who measured high TIA in Small White and Black Turtle Soup beans. Investigating protein availability, others (Koehler et al., 1987) found the best protein quality in Pinto beans, and the poorest in Black Turtle Soup and Red Kidney beans. Obviously, there exists a correlation with seed coat colour.

The trypsin inhibitor activity was higher in the yield produced under non-irrigated growing conditions, than under irrigated conditions. Independent of growing conditions and ripening time, the trypsin inhibitor activity was larger in soybeans than in beans (see Table 3).

The trypsin inhibitor activity was significantly lower in the selected white seeded bean lines in both non-irrigated and irrigated experiments, which may have a favourable influence on the quality of the food products. Irrigation did not have an effect on TIU levels in the non-selected coloured seeded bean lines. The significant differences in TIU levels among soya maturity groups could be revealed only in drought, and not under the conditions provided by irrigation.

Table 2

Nutritive quality of bean breeding lines with different coloured and sized seeds [Trypsin inhibitor amounts are expressed in trypsin inhibition units (TIU) per mg fat-free dry matter; fat and protein contents are expressed as a percentage of dry matter] 1992–1994

Breeding lines	Thousand grain weight (g)	TIU	Fat (%)	Protein (%)
White seeds				
Start P_1	231	15.42	1.73	29.90
R 86-139 P_2	170	13.71	2.07	30.83
D6-502/16	200	14.02	1.28	31.80
D6-501/18	194	11.75	2.53	31.99
D6-501/47	173	12.53	2.03	30.81
D6-802/2	177	20.23	1.61	29.81
D6-802/3	210	23.29	1.25	29.14
D6-902/9	206	16.36	1.49	29.73
D6-902/12	206	14.56	2.12	30.12
D5-1001/24	174	21.55	2.19	29.05
D5-1001/13	152	19.84	1.77	31.06
Mixed colours				
Coco	442	8.84	1.16	26.98
D830/1-17	510	14.72	1.33	26.28
D830/49	532	17.43	1.59	28.76
D830/55	547	12.26	2.71	27.09
Debreceni tarka/D-830/	506	16.36	1.63	24.97
Coloured seeds				
DRK USA	376	25.55	1.17	30.55
D-1076	447	21.08	0.98	29.38
D-1076/6	420	24.11	1.17	26.77
Nagykállói	344	20.60	1.76	28.04
D1175/20	300	21.41	1.32	30.41
D1175/6	310	26.10	1.36	31.00

Good digestibility could be achieved in soybean and bean seeds by selecting for low TIA under dry growing conditions. When such varieties are grown under irrigation they produce an even more favourable nutritive quality in the yield.

Because of the large fat content of soybeans, the seeds contained much more energy than bean seeds, where it varied between 1.25 and 1.95%. Within the maturity groups of soybeans, the fat content was only significantly different in the early and late maturity groups, regardless of the growing conditions.

Oil accumulation was greater in the mid-early soybean group grown under irrigation, than in the late maturing group. Irrigation did not increase the protein content in soybeans and beans, and there were no differences in protein content between the soybean maturity groups (Table 3).

Table 3

Effect of irrigation and years on the nutritive quality of different edible legumes
(A=without irrigation; B= irrigation; trypsin inhibitor activity is expressed in TIU;
fat and protein contents are expressed as a percentage of dry matter)

Group	Properties	TIU				Fat		Protein		
		A		A	B	A	B	A		B
		1992	1993	mean	1994	1993	1994	1992	1993	1994
BEAN	white-seeded	22.98	15.60^{d}	19.29	12.80*	1.95	1.68	28.76	–	28.73^{c}
	coloured seeded	20.99	15.67^{d}	18.33	15.89	1.69	1.25	29.84	–	24.10*
SOYBEAN	early maturing	35.99	70.00^{a}	53.00	18.75*	19.66^{a}	19.90^{a}	–	33.91	32.22^{b}
	mid-early maturing	32.93	57.00^{c}	44.97	29.25*	17.93^{b}	19.74*	–	34.74	35.69^{a}
	late maturing	34.59	62.50^{b}	48.55	24.41*	17.52^{b}	17.92^{b}	–	35.29^{a}	36.17^{a}
PEA	dry								24.44^{b}	–
$LSD_{5\%}$		5.09	5.09		5.09	0.46	0.46		1.87	1.87

Means within a column followed by the same letter are not significantly different to each other at the 5% level using Duncan's multiple range test.

* The difference between conditions A and B is significant.

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GENETIC STUDY OF CODEINE CONTENT BY DIALLEL ANALYSIS IN OPIUM POPPY (*PAPAVER SOMNIFERUM* L.)

K. LŐKÖS TÓTH[1], M. SZATMÁRI[2] and J. SUTKA[3]

[1]GENETICS AND PLANT BREEDING DEPARTMENT,
UNIVERSITY OF AGRICULTURAL SCIENCES, GÖDÖLLŐ, HUNGARY
[2]RESEARCH INSTITUTE FOR MEDICINAL PLANTS, BUDAKALÁSZ, HUNGARY
[3]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES,
MARTONVÁSÁR, HUNGARY

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The inheritance of codeine content in opium poppy (*Papaver somniferum* L.) was studied using a 5 × 5 full diallel cross. Both the general combining ability (GCA) and specific combining ability (SCA) were significant, indicating additive and non-additive gene action in the inheritance of codeine content. The reciprocal effect was neglected. The variance/covariance graphical analysis indicated the complete dominance of low codeine content. The variety Kompolti Resistant and the variety B-1 had the highest proportion of recessive genes, while the variety Kompolti had the largest number of dominant genes. In this set of opium poppy varieties the narrow sense heritability (h^2=0.44) was relatively low. It was concluded that codeine content was a quantitative character determined by several genes. In addition the variety B-1 appears to have a major gene for high codeine content.

Key words: opium poppy, *Papaver somniferum*, codeine, inheritance, diallel analysis

Introduction

The opium poppy is one of the most important Hungarian-produced medicinal plants. The pharmaceutical industry shows considerable interest in the quantity and quality of this plant. For this reason the principal goal in past breeding programmes was the enhancement of alkaloid content in the capsule, while the quantity and quality of the seed yield was only of secondary importance.

The process patented by Hungarian pharmacist János Kabay in 1931 made the extraction of alkaloids from the ripe capsule possible. The extract contains a large number of alkaloids. The six major alkaloids are the morphine alkaloids (morphine, codeine and thebaine; Bentley, 1970) and the isoquinoline alkaloids (narcotine, narcotoline and papaverine; Shamma, 1970; Bisset, 1985). Currently, the most significant industrial demand is for morphine, narcotine and codeine. Codeine is widely used with other agents as an anodyne and for catarrhal diseases of the respiratory system.

Several of the poppy varieties that are cultivated in Hungary accumulate large quantities of one alkaloid. For example the A-1 cultivar accumulates morphine, while Kék Gemona accumulates narcotine. However, there are no

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cultivars that simultaneously accumulate the three industrially important alkaloids, morphine, codeine and narcotine. The development of such a genotype is not an easy problem for breeders for two reasons. The biosynthetic pathways for these alkaloids are quite different from each other (Vágújfalvi, 1968). In addition, only limited knowledge is available on the inheritance of alkaloid accumulation. Morphine and codeine accumulation has been investigated in traditional plant breeding experiments and also in suspension cultures (Siah and Doran, 1991).

Hungarian research on poppies has focused on the physiology (Bernáth, 1979; 1989), morphology (Gracza and Sárkány, 1970), morphine content and stability parameters (Kálmán et al., 1989). Similar kinds of references can be found by foreign authors (Hofman and Menary, 1980; Ghiorghita et al., 1990; Bhandari and Gupta, 1991; Nigam and Patel, 1993).

Investigations on opium poppy with diallel analysis can be divided into two groups on the basis of the plant traits studied. Morpho-phenological studies tried to clarify questions involving the inheritance of flowering and ripening time, capsule size and number, plant height, peduncle length, petal size, seed mass and harvest index (Briza and Hlavackova, 1983; Saini and Kaicker, 1983; Saini, 1988; Sharma et al., 1988; Singh and Khanna, 1991; Kandalkar et al., 1992; Shukla et al., 1992). The results of these crossing studies have been very significant in the development of theoretical approaches and breeding practice.

Another group of published papers deals with the correlations between the above traits and raw opium (latex) and morphine content. These studies analysed the inheritance of latex and morphine content with diallel analysis systems of different sizes and structures (Briza, 1983; Khanna and Shukla, 1989; Lal and Sharma, 1990; Mishra and Barche, 1991). Lal and Sharma (1991) examined the simultaneous inheritance of morphine, codeine, thebaine and narcotine. The results showed a significant and prevailing dominance in the parents for latex yield and morphine, codeine, thebaine and narcotine content. However, the additive component was also significant for the morphine, codeine and narcotine contents. The investigations were especially valuable because the results provided useful information for collecting parental material for breeding programmes.

This study analyses the inheritance of codeine content based on generally available varieties. The inheritance of morphine content, based on the same material, has already been reported (Tóthné et al., 1994). A further aim is to work out a new variety breeding programme to enhance codeine content, making use of the results of this genetic analysis.

Materials and methods

Five opium poppy varieties (1. Kompolti, 2. T-2, 3. B-1, 4. Kék Duna, 5. Kompolti Resistant) were used as parents. The parental crossings (in 1992) and the cultivation of the F_1 generations were performed in the field nursery of the Genetics and Plant Breeding Department, University of Agricultural Sciences, in Gödöllő. The 20 possible F_1 crosses were made among the five varieties. The experimental unit was one row of 10 plants, replicated twice. The rows were 40 cm apart and the plots were bordered by three plants at each end. The capsules produced by one row of plants were combined for analysis of codeine content by liquid chromatography at the Research Institute of Medicinal Plants.

The data were analysed with descriptive statistical methods (average, variance, CV %). Combining ability was determined by the Griffing-1 method (1956). The variance (V_r) and covariance (W_r) were determined by Hayman's method (1954).

Results and discussion

Description of varieties and their combinations

The average codeine content for the two replicate samples of the five parents, their F_1 progenies, the mean, variance and CV% values of the maternal and paternal combinations are presented in Table 1. The analysis of variance for the twenty-five genotypes was significant at the 5% level. Content differences greater than 0.52‰ were significant.

These results show that the B-1 variety had significantly higher codeine content (1.36‰) than the others and that the Kompolti Resistant parent had the lowest content (0.25‰, Table 1). The mean of the five parental genotypes was 0.6‰, the standard deviation was 0.2‰ and the coefficient of variation was 31%. Although the genotypes were originally chosen for morphine analysis, these data show that they are suitable for the diallel analysis of codeine content.

Griffing's combining ability analysis

The variance analysis of combining ability is given in Table 2. All the F-tests are significant but the magnitudes of the effects are different. The high F-value for general combining ability (GCA) is due to the additive polygene

Table 1

Mean data of codeine contents of parents and their combinations in the F_1 generation

Parents	1	2	3	4	5	$\bar{x}$	s	CV%
1	0.44	0.29	0.45	0.56	0.68	0.48	0.15	30.1
2	0.61	0.57	0.47	0.38	0.37	0.48	0.11	22.6
3	0.48	0.48	1.36	0.28	1.23	0.77	0.49	64.2
4	0.45	1.55	1.41	0.40	0.29	0.82	0.61	74.1
5	0.56	0.25	1.35	0.58	0.25	0.60	0.45	75.2
$\bar{x}$	0.51	0.63	1.01	0.44	0.56			
s	0.07	0.53	0.50	0.13	0.41			
CV%	14.6	84.7	49.7	28.9	72.5			

Table 2
Analysis of variance for combining ability

Source of variation	d.f.	S.S.	M.S.	F.
GCA	4	0.91	0.23	3.8**
SCA	10	1.52	0.15	2.5**
RE	14	1.44	0.14	2.3*

*, **: significant at P=0.05, P=0.01, respectively

system. The F-value of the specific combining ability (SCA) is lower and due only to parent B-1 and the high F_1 value for Kék Duna × T-2. According to general combining ability effects parent B-1 was a good combiner for codeine content. The F-value for reciprocal effect (RE) is just above the 5% significance level and due almost entirely to the F_1 values for the fourth parent. For this reason, the reciprocal effect was neglected in the graphical analysis.

Graphical analysis of relation of variance (V_r) and covariance (W_r)

The presence of the additive polygene system was confirmed by the analysis of parental variance and parent-offspring covariance (Fig. 1). The regression coefficient (b) is not significantly different from 1. This indicates that non-additive genetic variation is present as dominance only. The regression line crosses the covariance axis near –0.019, indicating a complete dominance for the low codeine content.

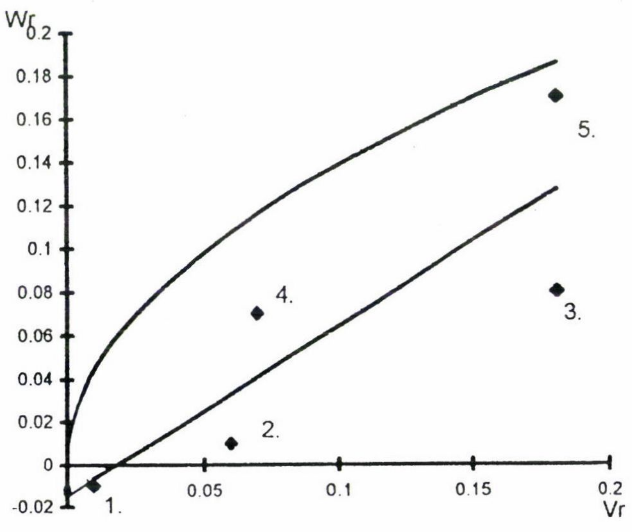

Fig. 1. Graphical analysis of codeine content in F_1 generation (parabola: Wr = 0.19 V; regression line: W = 0.788V–0.019)

The relatively low narrow sense heritability (h^2=0.44) is attributable to the lower additive effect compared to the dominant effect. The five parental genotypes were uniformly distributed along the regression line. The relatively high values of W_r and V_r show that the parent Kompolti Resistant had the highest proportion of recessive genes. The parent Kompolti contained the largest number of dominant genes because its W_r and V_r values were closest to the origin. On the basis of the results it was concluded that the inheritance of codeine content involved multiple genes that were mostly additive in their effect. This finding confirms earlier results (Lal and Sharma, 1991). The variety B-1, which had the highest codeine content, proved to be a good combiner, and appeared to have a major gene for codeine content.

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CYTOMORPHOLOGICAL STUDIES IN TRITICALE IN RELATION TO GRAIN SHRIVELLING

V. K. KHANNA, M. R. RAO and R. R. KOMARNENI

DEPARTMENT OF GENETICS AND PLANT BREEDING, G.B. PANT AGRIVARSITY, PANTNAGAR 263145, INDIA

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Three cultivars of hexaploid triticale, namely UPT 72142 having shrivelled grains, UPT 79339 with medium grains, and UPT 79354 having plump grains, were used for cytological and morphological studies in relation to grain shrivelling. Giemsa staining revealed that in UPT 79354, with plump grains, and UPT 79339, with medium grains, there were only four rye chromosomes, while UPT 72142, with highly shrivelled grains, possessed 10 rye chromosomes. The endosperm was single-layered in UPT 79339 in the central ventral region, but double-layered in other regions. In UPT 79354 the central portion was full of endosperm cells with a lumen in the centre. The crease was the widest and the endosperm region the smallest in the highly shrivelled UPT 72142 as compared to the other strains. The highly shrivelled appearance of the grains of UPT 72142 seemed to be due primarily to pericarp shrivelling and to separation from the seed coat.

Key words: hexaploid triticale, Giemsa staining, microtomy, grain shrivelling

Introduction

Triticale is a "man-made" cereal crop. It is a new genus, the result of crossing wheat (genus *Triticum*) with rye (genus *Secale*). The name triticale is formed from the Latin names of the two parental genera. On the production side, triticales can, under high fertility conditions, now yield as well as bread wheat. As regards protein content, triticale is also potentially important as it generally has a greater dry weight protein percentage than wheat. Moreover, this contains a higher proportion of lysine. In addition to its increased carbohydrate and protein yield potential as compared with wheat and rye, there are other triticale characteristics which are even more critical in justifying the development of this crop. These are its qualities of tolerance to cold weather, drought conditions, poor, acid or sandy soils and resistance to disease. However, its commercial exploitation remains a problem because of the high degree of grain shrivelling. Intensive breeding has failed to overcome the problem, suggesting that grain shrivelling is the result of a complex genetic interaction. It is thought that abnormalities due to genetic causes may manifest themselves very early in the developing grain, namely, in the first few weeks after fertilization. These abnormalities could be associated with increased or decreased mitotic activity, meiotic instability, faulty endosperm development or telomeric rye heterochromatin. Bennett (1977) proposed that the genome incompatibility of

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triticale was mainly caused by the presence of large blocks of late-replicating heterochromatin on the telomeres of rye chromosomes, but not on wheat chromosomes (Gill and Kimber, 1974). This heterochromatin is correlated with seed abortion and with grain shrivelling at maturity (Bennett and Gustafson, 1982; Varghese and Lelley, 1983). Grain shrivelling may be due to the slow growth of the endosperm or the appearance of cavities in the endosperm, both of which limit the capacity of the grain to accumulate and store carbohydrates and proteins (Thomas et al., 1980). According to Srivastava (1978), grain shrivelling in triticale may be related to a decreased accumulation of starch, which in turn may be due to reduced biosynthesis or increased degradation.

The objective of the present study was to investigate the effect of rye chromosome substitutions on grain improvement, to study the morphological changes at different stages of grain development and to correlate these with grain shrivelling in hexaploid triticales. This would also help us in the selection of parental materials for hybridization in our grain improvement programme for triticale.

Materials and methods

The experimental material comprised three cultivars of hexaploid triticale (*Triticosecale*, Wittmack), namely UPT 72142 having shrivelled grains, UPT 79339 with medium grains, and UPT 79354 having plump grains. Seeds were placed on moist filter paper in Petri dishes and germinated for about 36 h in an incubator at 25°C. After 36 h, 12 cm long root tips were taken and treated with 0.2% colchicine for about 8 h and then washed thoroughly 3–5 times with distilled water. They were placed in 1:3 acetoalcohol for 24 h and then transferred to 70% alcohol and stored in a refrigerator. For making slides the root tips were treated with 5% enzyme mixture (2.5% cellulase + 2.5% pectinase) for 45 min at 25°C and then rinsed in distilled water. The enzyme-treated root tips were directly squashed in acetocarmine and then air sealed with a rubber solution. The cover slips were slipped off using liquid nitrogen, then the slides were air dried for 24 h. The slides were placed in 5% barium hydroxide for 5 min and then washed with hot distilled water 5 times and placed in 2 × SSC (0.3 M sodium chloride + 0.03 M sodium citrate) at 60°C for 1 h. They were then washed in distilled water and placed in phosphate buffer (pH 6.8) for 3 min to achieve uniform staining. Staining was carried out in 1.5% Giemsa solution for 20 min at room temperature. The slides were then air dried and mounted in neutral balsam. The method of Gill et al. (1981) was followed for chromosome identification.

For morphological studies on the developing grains, plants were grown in a randomized block design, with 3 replications. As the heads emerged, each head was bagged and the anthesis date was recorded. At 5 and 35 days post-anthesis, two spikes from the main tiller were harvested and fixed in formaldehyde-propionic acid –70% ethanol (FPA fixative). The material was fixed for 2 h and then preserved in 70% ethanol. Dehydration, infilteration and embedding were performed according to the ethanol-tertiary butanol paraffin schedule of Sass (1958). Transverse sections (15 μm thick) were prepared on a Spencer 815 rotary microtome and stained with toluidine blue, and permanent slides were prepared with neutral balsam. Cross-sections were viewed and photographed with a Leitz research microscope equipped with an automatic camera.

Results

Giemsa staining revealed that in UPT 79354, with plump grains, and UPT 79339, with medium grains, there were only four rye chromosomes, whereas UPT 72142, with highly shrivelled grains, had 10 rye chromosomes (Komarneni and Khanna, 1989).

The endosperm was single-layered in UPT 79339 in the central ventral region, but double-layered in other regions (Fig. 1c). In UPT 79354 the central portion was full of endosperm cells with a lumen in the centre (Fig. le). At 5 days post-anthesis, the mass of cellularized endosperm did not fill the endosperm cavity. This characteristic was more pronounced in grains of UPT 79339, with medium shrivelled grains (Fig. 1c), which showed a large lumen in the centre. In plump-grained UPT 79354 (Fig. 1e) this lumen was very small and the endosperm cavity was nearly completely filled with starchy endosperm cells. In UPT 72142, having shrivelled grains, though the lumen was very small, at the same time the endosperm region inside the aleurone layer was also very small (Fig. 1a). The best developed cells could be seen in the plump-grained UPT 79354. The crease area was the widest and the endosperm region the smallest in the highly shrivelled grains of UPT 72142, as compared to the other strains.

At 35 days post-anthesis the grains showed surface depressions in UPT 72142, and the pericarp and the seed coat had collapsed to produce a wrinkled appearance (Fig. 1b). Grains of UPT 79354 (Fig. 1f) and UPT 79339 (Fig. 1d) showed the same abnormal characteristics, but to a lesser extent than those of UPT 72142.

Discussion

Rye chromosomes have often been correlated with grain shrivelling in triticale. Genome incompatibility in triticale was mainly caused by the presence of large blocks of late-replicating rye chromosomes (Bennett, 1977). This results in slow growth of the endosperm or incomplete filling of the endosperm cells with carbohydrates and proteins (Thomas et al., 1980) due to reduced biosynthesis or increased degradation (Srivastava, 1978) resulting in shrivelled grains. Rye chromosomes are mostly found to become substituted by the D-genome chromosomes of wheat (Merker, 1976). The Giemsa banding technique specifically stains portions of chromosomes containing repetitive DNA, which is present in rye chromosomes. This helps in recognizing individual chromosomes.

Giemsa staining revealed that in UPT 79354, with plump grains, and UPT 79339, with medium grains, there were only four rye chromosomes (Komarneni and Khanna, 1989). In these two strains the number of rye chromosomes is lower and there is therefore less heterochromatin, resulting in better grains.

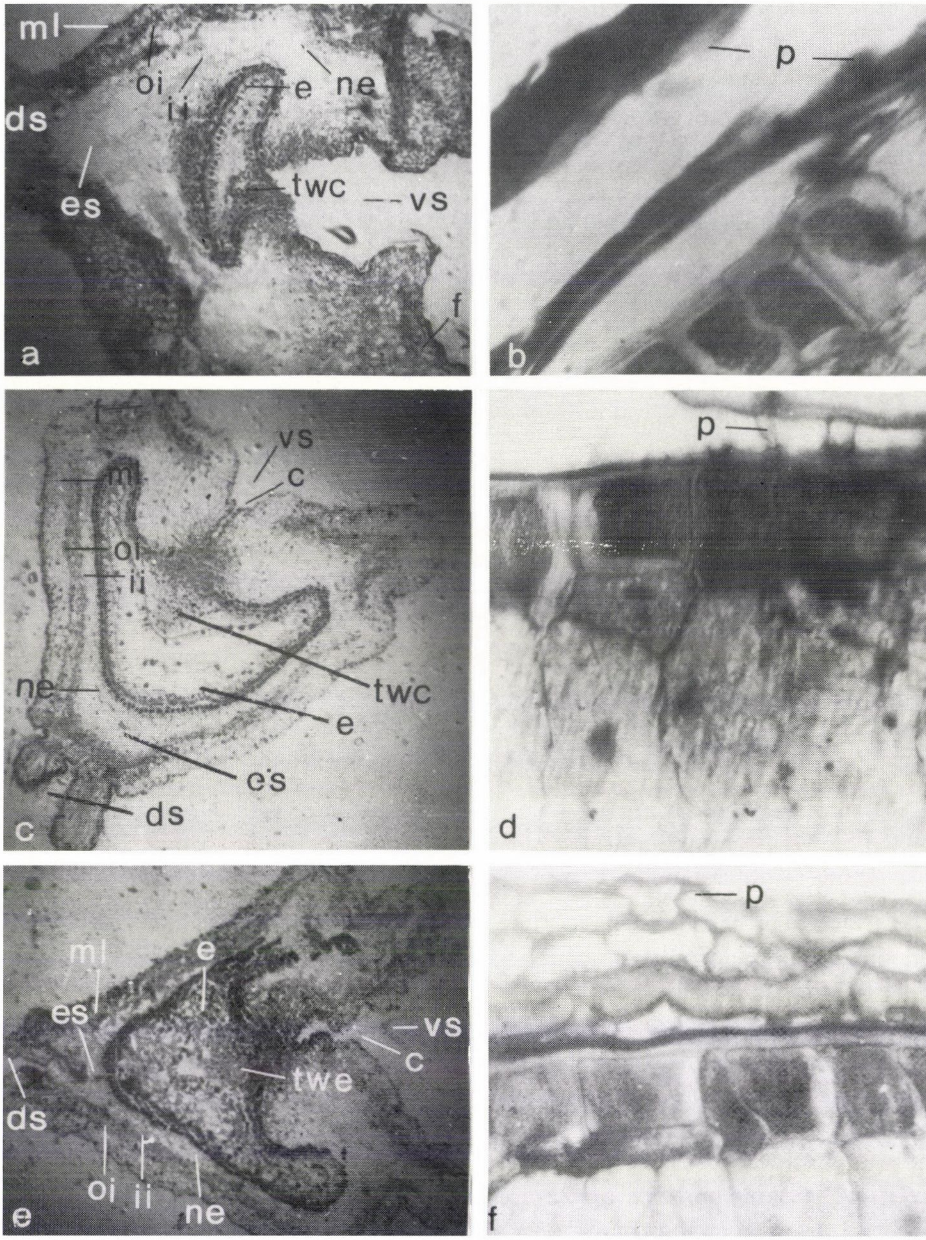

Fig. 1. Cross-sections of triticale grains at several developmental stages.

(Oi = outer integument, ii = inner integument, p = pericarp, ne = nucellar epidermis, al = aleurone, e = endosperm, c = crease, ml = meristematic layer, twc = thick-walled cells, f = flank, vs = ventral side, ds = dorsal side, es = empty space). *a.* Highly shrivelled UPT 72142 at 5 days post-anthesis (7×4). *b.* UPT 72142 at 35 days post-anthesis (7×40) showing separation of the pericarp from the rest of the grain. *c.* Medium shrivelled UPT 79339 at 5 days post-anthesis (7×4). *d.* UPT 79339 at 35 days post-anthesis (7×40). *e.* Plump-grained UPT 79354 at 5 days post-anthesis (7×4). *f.* UPT 79354 at 35 days post-anthesis (7×40)

The lower amount of heterochromatin leads to a reduction in the formation of aberrant nuclei during endosperm formation and consequently to a reduction in kernel shrivelling (Bennett and Gustafson, 1982). UPT 72142, with highly shrivelled grains, had 10 rye chromosomes (Komarneni and Khanna, 1989).

The endosperm lies within the embryo sac, which is surrounded by several layers of nucellar tissue, integument and pericarp. This was single-layered in UPT 79339 in the central ventral region, but double-layered in other regions (Fig. 1c). In UPT 79354 the central portion was full of endosperm cells with a lumen in the centre (Fig. 1e). Subsequent endosperm development resulted from cell enlargement and the deposition of starch and protein, as suggested by the clear aleurone layer surrounding the endosperm, which could be seen even on the fifth day post-anthesis. At 5 days post-anthesis, the mass of cellularized endosperm did not fill the endosperm cavity. This characteristic was more pronounced in grains of UPT 79339, with medium shrivelled grains (Fig. 1c), where there was a large lumen in the centre. In plump-grained UPT 79354 (Fig. 1e) this lumen was very small and the endosperm cavity was nearly completely filled with starchy endosperm cells. In UPT 72142, having shrivelled grains, though the lumen was very small, at the same time the endosperm region inside the aleurone layer was also very small (Fig. 1a). The best developed cells could be seen in the plump-grained UPT 79354. The crease area was the widest and the endosperm region the smallest in highly shrivelled UPT 72142, as compared to the other strains. This could be the result of early developmental problems (Bennett, 1977; Kaltsikes et al., 1975).

The pericarp is attached to the grain. The highly shrivelled appearance of the grains of UPT 72142 seemed to be due primarily to pericarp shrivelling and separation from the seed coat (Fig. 1b). This may result in a greater number and depth of depressions in these grains. The endosperm depressions may be caused by a collapse of the nucellar epidermis and seed coat into an insufficiently filled portion of the starchy endosperm, perhaps dependent on how the starchy endosperm was packed. At 35 days post-anthesis the grains showed surface depressions, and the pericarp and the seed coat had collapsed to produce a wrinkled appearance (Fig. 1b). Grains of UPT 79354 (Fig. 1f) and UPT 79339 (Fig. 1d) showed the same abnormal characteristics but to a lesser extent than in those of UPT 72142.

Our observations, along with earlier findings by Bennett (1977) and a review of the developmental aspects of grain shrivelling in triticale (Thomas et al., 1980), lead us to conclude that grain shrivelling in secondary hexaploid triticale has its origin in incompatibility problems between wheat and rye chromosomes in the triticale genotype. The extent of grain shrivelling is established at the earliest stages of endosperm development when aberrant nuclei are formed. As a consequence, the number of cells that remain at maturity is reduced. This leads to an early termination of dry matter accumulation because no metabolic sink area for storage exists. Consequently, physiological maturity occurs early. Because the embryo sac is designed to be completely filled by the expanding endosperm cells and cannot be filled when the number of endosperm cells is reduced, the pericarp, seed coat and aleurone layers collapse into the empty spaces during the later stages of grain maturation. The final result is endosperm voids and depressions that may disrupt the aleurone cells, facilitating enzymatic attack (primarily alpha-amylase) on the starch granules in adjacent cells, and the pericarp shrivelling that characterizes most mature triticale grains.

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INCORPORATION OF ^{15}N-LABELLED FERTILIZER NITROGEN INTO WHEAT GRAIN PROTEINS DURING GRAIN DEVELOPMENT

K. BERECZ, K. DEBRECZENI and I. RAGASITS

INSTITUTE OF AGRONOMY, PANNON UNIVERSITY OF AGRICULTURE, GEORGIKON FACULTY, KESZTHELY, HUNGARY

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The aim of our experiments was to study the incorporation of ^{15}N-labelled fertilizer nitrogen into winter wheat *(Triticum aestivum* L.*)* grain and its protein fractions during grain development. The microplot N fertilization experiments were carried out on a eutric Cambisol of medium N status in Keszthely (Hungary). From the time when grain moisture was 70% to full ripening, six samples were taken; the grains of 100 spikes were hand-threshed each time. Grain moisture, 1000-grain-weight, total N and ^{14}N to ^{15}N ratios in grains and in their salt- and ethanol-soluble protein fractions were determined. N doses of 60 or 120 kg ha^{-1} (the latter applied single or split) distributed during the spring, in addition to autumn base N-fertilizer of 80 kg ha^{-1}, did not increase the amount of N in the grains. However, fertilizer-N, which is more readily available to plants than soil-N, was incorporated into the grains in proportions of 11–26%. The amounts of N incorporated into the ripe grains and their salt-and ethanol-soluble fractions from the second 60 kg ha^{-1} N dose, applied at plant developmental stage Feekes 6, were 61, 35 and 69% higher, respectively, than those incorporated from the first N dose of the same quantity, given at Feekes 2–3. As a result of doubling the 60 kg ha^{-1} N dose applied at tillering, the amount of fertilizer-N detected in the grains rose more than twofold. At 70% grain moisture the salt-soluble protein fraction already contained 63–75% of the nitrogen originating from spring doses and later detected in this fraction of the ripe grains. In the gliadin fraction, this proportion amounted to only 9–11%.

Key words: grain development, grain proteins, nitrogen incorporation, ^{15}N field experiment, *Triticum aestivum* L., wheat

Introduction

Nitrogen is one of the most important elements, as its availability influences the synthesis, accumulation and composition of proteins in developing wheat kernels (Schipper and Jahn-Deesbach, 1981; Stenram et al., 1990). If we want to make the best use of nitrogen fertilization in order to raise the grain protein content and improve its composition from the point of view of baking quality, we need a better knowledge of the extent of fertilizer-N incorporation into the wheat grain and its distribution in the protein fractions.

The utilization of nitrogen fertilizer in the ripe grain has been investigated using the ^{15}N tracer technique by several authors (Varga et al., 1973; Christensen and Meinst, 1982; Kiss et al., 1985; Paredes-López et al., 1985). Experiments making use of the N isotope method to reveal the dynamics of N

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incorporation into the grain and its protein fractions throughout grain development have scarcely been reported. However, such information is essential to provide a theoretical basis for intervention through directed nutrient supplies into the development of the wheat grain with the aim of quality improvement.

Baking quality is mainly determined by the quantity and quality of gluten proteins (MacRitchie, 1973; Pallagi-Bánkfalvi, 1984; Branlard and Dardevet, 1985). Understandably, this fact has attracted considerable attention to investigations on the effects of N fertilization on quantitative and qualitative changes in the gliadin and glutenin fractions (Timms et al., 1981; Kemény et al., 1991; Scheromm et al., 1992; Peltonen and Virtanen, 1994).

There are also studies, however, which point to the role of water- or salt-soluble protein fractions in baking quality. When studying a world wheat collection, Preston et al. (1992) found that the salt-extractable "Osborne" proteins of the flour are in close negative relationship with the farinograph dough strength parameters. Chen and Hoseney (1995) reported that the water-soluble flour fraction causes sticky dough. Because the effect of these fractions on the technofunctional properties of wheat flour appears to be far from negligible, it is worthwhile studying the effect of N fertilization on the accumulation of the salt-soluble protein fraction, too.

Therefore, in a harvesting study based on an N fertilization experiment, the incorporation of ^{15}N-labelled fertilizer nitrogen was investigated in winter wheat grains and their salt- and ethanol-soluble "Osborne" protein fractions.

Materials and methods

Field cultures and ^{15}N-treatments

A one-year N fertilization experiment was carried out on a eutric Cambisol of medium N status in Keszthely (Hungary) with the winter wheat *(Triticum aestivum* L.*)* variety "Martonvásári 16". In a 45 m^2 plot four microplots each measuring 1 m^2 were marked out without replications. 80 kg ha^{-1} unlabelled N, 65.5 kg ha^{-1} P and 124.5 kg ha^{-1} K were applied to the 45 m^2 plot before sowing. In addition to the base fertilization, three microplots were N-topdressed, partly or entirely with N-labelled fertilizer, at plant developmental stages Feekes 2–3 and 6 (Brouwer, 1970), i.e. at the beginning of tillering and shooting, as presented in Table 1. The labelled fertilizer (9.3 atom% N) consisted of four parts of NH_4NO_3 labelled both in the cation and in the anion and one part of N-labelled urea. The fertilizer was applied in liquid form between the rows.

Sampling

Between a grain development stage of 70% moisture and full ripening six harvests were made at weekly intervals (Table 1). To compensate for the lack of field replications, 100 spikes per plot were cut from each microplot on each occasion. The grains of the spikes were hand-threshed immediately after harvest.

Table 1
Treatments used in the experiment and grain moistures (%) pertaining to the different sampling dates

Treatment ($kg\ N\ ha^{-1}$)			Sampling dates					
	Feekes stage							
No.	2–3	6	17. 06	24. 06	01. 07	08. 07	15. 07	23. 07
1	–	–	70.1	58.7	47.1	35.6	18.5	17.3
2	60[a]	–	70.9	58.5	45.8	33.9	19.9	17.5
3	60[b]	60[a]	69.5	57.6	46.3	34.3	19.9	17.2
4	120[a]	–	70.5	56.1	46.7	29.0	19.8	16.9

a: nitrogen fertilizer containing 9.3 atom % ^{15}N, b: unlabelled N-fertilizer.
In each treatment base fertilization with 80 kg ha^{-1} of unlabelled nitrogen was applied before sowing.

Measurements

On the day of sampling, grain moisture and total nitrogen contents were measured. The ^{15}N to ^{14}N ratios within the grain nitrogen content were also determined. A part of the samples was dried at air temperature for the determination of 1000-grain-weight. The remaining samples were stored at –18°C for protein fractionation. The nitrogen data are expressed on a single grain basis. In this study in place of the botanically more appropriate term 'kernel', the word 'grain' is used, since this is predominantly employed in the related literature.

Protein fractionation and nitrogen analysis

After milling the freeze-dried grain samples a classic Osborne's extraction was performed on the basis of the fractionation method of Pochinok (1976). Total nitrogen content and ^{15}N to ^{14}N ratios in fractions extracted with 5% K_2SO_4 and 70% ethanol were determined. Total nitrogen was assayed with Kjeldahl's method as modified by Lengerken et al. (1974). For the isotope ratio measurements, the ammonium in the Kjeldahl digest was steam-distilled into 0.05N HCl and the distillate was analysed with an emission spectrometer (ISONITROMAT 5201 made by STATRON, East Germany).

Results and discussion

Total N in grains

Figure 1 shows the accumulation of nitrogen during grain development after the different N treatments. None of the spring N doses applied in addition to the autumn base N fertilizer increased the total nitrogen content calculated for one kernel. The ripe grains of the control and the spring top-dressed plots, all contained approximately the same amount (750–740 μg) of nitrogen. Consequently, on Ramann's brown forest soil with medium N status a base fertilization of 80 kg ha^{-1} appears to be sufficient for the grains to reach the

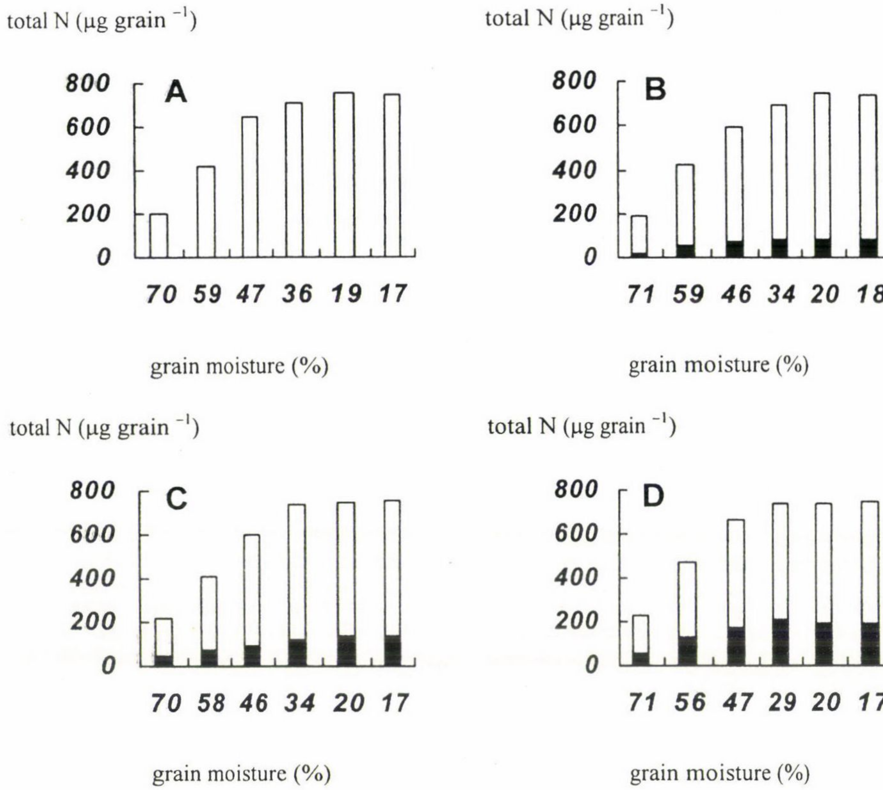

Fig. 1. Accumulation of total ^{15}N-labelled fertilizer nitrogen in the developing wheat grain (Legend: A, B, C and D represent treatments 1, 2, 3 and 4, respectively; for treatments see Table 1. The dark portion of the bars depicts the amount of labelled fertilizer nitrogen within the total grain N-content)

highest nitrogen content. (The maximum N concentration, expressed as a percentage of dry weight, was obtained with the single 120 kg dose in treatment 4.) The effect of N fertilization may have appeared in the increase of protein yield depending on dry weight production. This was not examined directly, because it could only have been detected if a single sampling had been made at full maturity.

Incorporation of ^{15}N labelled fertilizer nitrogen into grains

As already stated above, the total nitrogen in the grains from the plot that had only been base-fertilized before sowing did not differ from that in the grains from top-dressed plots. In spite of this fact, 11–26% of the total nitrogen assimilated by the grains originated from the labelled fertilizer applied during the spring and not from the soil. Apparently there was a difference in the availability of the two N sources. The ratios shown here are lower than those reported by Varga et al. (1973) and Parades-López et al. (1985). However, among others things, the different proportions of nitrogen incorporation from different fertilizer sources and doses shown by the authors cited make comparisons difficult.

The incorporation of ^{15}N-labelled fertilizer nitrogen was observed in two main phases with different rates, as in the case of total nitrogen. The first phase, with the higher rate for both total and ^{15}N-labelled fertilizer nitrogen, lasted up to a grain moisture of 29–36%. Grains with 70% moisture contained 26–30% of the nitrogen measured in the ripe grains, irrespective of whether the total or the labelled fertilizer nitrogen was examined. Sowers et al. (1994) reported a high correlation between wheat grain N concentration at maturity and grain N concentration at the late milky and floury stages, for a wide range of final grain protein contents. The present results confirm that N accumulation during this grain developmental phase is decisive for the final amount of N in the ripe grains.

From the 60 kg N dose applied later, in addition to the first early spring dose of the same quantity, the amount of ^{15}N-fertilizer nitrogen incorporated into the grains was 61% higher. The grains were able to assimilate the nitrogen applied at a later developmental stage to a greater extent. The later the N-dose was applied, the higher extent of incorporation into the grains was detected also in other studies, not only for wheat (Enikov and Rajkova, 1979; Kiss et al., 1985; Harms and Nowak, 1990) but also for maize (Ta and Weiland, 1992). As a result of doubling the first 60 kg spring dose, the amount of ^{15}N-fertilizer nitrogen detected in the grains rose more than twofold. With this treatment the nitrogen incorporated into the grains from the labelled fertilizer amounted to more than a quarter of the total N amount measured in the grains.

Nitrogen incorporation into the fraction extracted by K_2SO_4 solution

Table 2 contains the data for nitrogen incorporation into the fraction extracted by 5% K_2SO_4, including non-proteins, albumins and globulins. The dynamics of the N incorporation into this fraction was similar in the different treatments. The accumulation continued practically up to the third sampling date, that is up to a grain moisture of 46–47%. Grains with 70% moisture already contained a major part, 70–80%, of the N amount measured in this protein fraction in the ripe grains. The ^{15}N fertilizer nitrogen incorporated into this fraction represented a smaller proportion, amounting to 63–75% in the different treatments. These data show that the incorporation dynamics of total and ^{15}N fertilizer nitrogen into the salt-soluble grain protein fraction were similar.

In the literature contradictory reports can be found regarding the effect of N fertilization on these fractions. Wu and McDonald (1976) reported a

Table 2

Nitrogen accumulation in the salt-soluble protein fraction of the developing wheat grain. The fraction containing the non-proteins, albumins and globulins was extracted with 5% K_2SO_4

Treatment No.	Grain moisture (%)	Total nitrogen (μg grain^{-1})	^{15}N fertilizer nitrogen (μg grain^{-1})	Proportion of ^{15}N fertilizer nitrogen in total nitrogen (%)
1	70.1	143		
	58.7	177		
	47.1	205		
	35.6	196		
	18.5	204		
	17.3	195		
2	70.9	139	14.5	10.4
	58.5	184	19.5	10.6
	45.8	197	18.9	9.6
	33.9	203	21.7	10.7
	19.9	201	22.9	11.4
	17.5	196	22.9	11.7
3	69.5	152	23.3	15.3
	57.6	174	27.7	15.9
	46.3	196	32.5	16.6
	34.3	217	34.1	15.7
	19.9	204	30.0	14.7
	17.2	203	31.9	15.7
4	70.5	162	39.5	24.4
	56.1	198	48.7	24.6
	46.7	211	46.6	22.1
	29.0	182	45.9	25.2
	19.8	190	51.3	27.0
	16.9	193	54.0	28.0

significant increase in the content of soluble proteins and non-proteins in flour samples as an effect of N fertilization. Abrol et al. (1971) and Doekes and Wennekes (1982) could not detect any appreciable changes in the albumin or globulin contents of the grain or flour samples. By contrast, Kolbe and Müller (1983) detected increases in both fractions as an effect of increasing N doses. In the present experiments, an increasing rate of ^{15}N incorporation into the fraction containing the non-proteins, albumins and globulins could be detected, although split or single spring doses did not increase the amount of salt-soluble protein-N in the ripe grains as compared with base fertilization before sowing. The proportion of ^{15}N fertilizer nitrogen related to total nitrogen in this protein fraction was similar to that in the total grain protein.

From the second spring N dose of 60 kg applied in addition to an early spring dose of the same quantity, the amount of N incorporated into the salt-soluble fraction of the mature grains was 35% higher, so the proportion of ^{15}N

fertilizer nitrogen within the total N contained in this fraction increased from 10.7% to nearly 16%, averaged over the samplings. As a result of doubling the early spring dose of 60 kg, the amount of ^{15}N fertilizer nitrogen detected in the K_2SO_4-soluble fraction rose more than twofold, just as in the total grain protein. Enikov and Rajkova (1979) detected only small changes in the proportion of ^{15}N-labelled fertilizer incorporated into the albumin fraction at maturity when they applied labelled N fertilizer in the autumn or at five different times in spring up to milky ripening. Non-proteins showed both increases and decreases, while globulin somewhat increased as a result of topdressing.

Nitrogen incorporation into the ethanol-soluble fraction

Data for N incorporation into the gliadin fraction extracted with 70% ethanol are summarized in Table 3.

Table 3

Nitrogen accumulation in the gliadin fraction of the developing wheat grain. The fraction was extracted with 70% ethanol

Treatment No.	Grain moisture (%)	Total nitrogen (μg grain^{-1})	^{15}N fertilizer nitrogen (μg grain^{-1})	Proportion of ^{15}N fertilizer nitrogen in total nitrogen (%)
1	70.1	26		
	58.7	156		
	47.1	272		
	35.6	319		
	18.5	338		
	17.3	333		
2	70.9	26	2.8	10.6
	58.5	162	15.7	9.7
	45.8	261	29.2	11.2
	33.9	322	36.1	11.2
	19.9	330	29.7	9.0
	17.5	320	31.7	9.9
3	69.5	35	5.6	16.0
	57.6	153	26.2	17.1
	46.3	261	42.0	16.1
	34.3	330	54.1	16.4
	19.9	316	49.6	15.7
	17.2	335	–	–
4	70.5	35	7.2	20.5
	56.1	180	48.8	27.1
	46.7	288	72.9	25.3
	29.0	345	70.0	20.3
	19.8	332	74.4	22.4
	16.9	338	88.2	26.1

The amounts of both total and ^{15}N fertilizer nitrogen detected in the gliadin fraction showed a more rapid increase at the beginning of grain development as compared with that of the salt-soluble fraction. By full maturity, the amount of total N detected in the ethanol-soluble fraction at the grain development stage with 70% moisture content rose 9–13-fold in the different treatments. The increase in ^{15}N fertilizer nitrogen per grain was similar: 9–11-fold.

The dynamics of incorporation of both total and labelled fertilizer nitrogen also appeared to be similar in the case of the gliadin fraction, i.e. the incorporation was very rapid at the beginning of grain development.

As compared to the albumins and globulins, changes in the gliadin concentration are more markedly dependent on the N supply, as reported by several authors (Günzel, 1962; Abrol et al., 1971; Doekes and Wennekes, 1982). The data of N analysis in the present experiment, as calculated on a single grain basis, show more rapid N incorporation into the gliadin fraction as a result of increasing N doses, but the amount of total gliadin-N per grain did not change substantially.

From the second 60 kg N dose applied at early shooting, in addition to the first dose of the same quantity, the amount incorporated into the gliadin fraction of the ripe grains was 69% higher. As a result of doubling the early spring dose, the amount of ^{15}N-fertilizer nitrogen detected in the gliadin fraction rose more than twofold, just as was concluded in the case of the salt-soluble fraction and total grain protein. Enikov and Rajkova (1979) could also detect a higher proportion of ^{15}N in the gliadin fractions at maturity, incorporated from later or higher spring N doses.

The proportion of N fertilizer nitrogen within the total N contained in the gliadin fraction was very similar to that of the salt-soluble protein fraction or total grain protein.

Distribution of protein fractions during grain development

Figure 2 shows the distribution of protein fractions within the total protein content per grain in the second treatment. Similar figures could also be presented for the other treatments. Abundant literary data exist to show that N fertilization can change the distribution of the fractions in grain proteins (Abrol et al., 1971; Kolbe and Müller, 1983; Stenram et al., 1990). These results are, however, unclear regarding the trend of the changes as a proportion of the different fractions. From the results of a single crop-year and two fractions, far-reaching conclusions cannot be drawn, but the pattern of N incorporation can be demonstrated.

As seen in Fig. 2, at the beginning of grain development the incorporation of both total and ^{15}N fertilizer nitrogen was proportionally greater in the salt-soluble fraction than in the gliadins. Later, the proportion of both total and ^{15}N

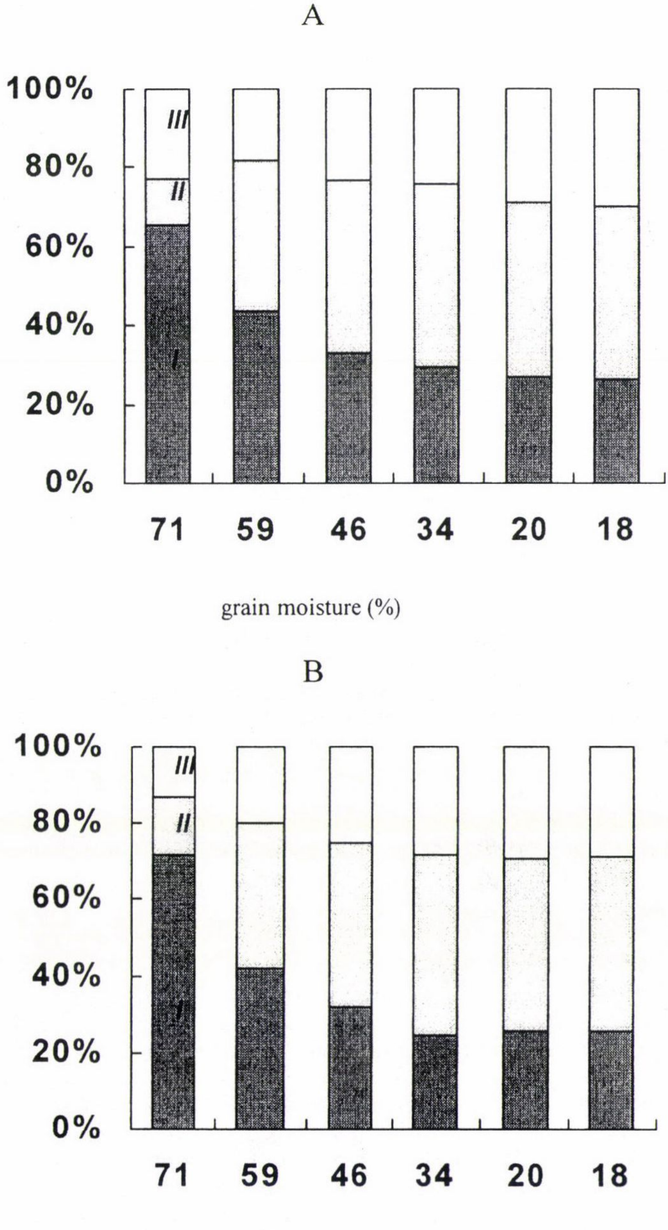

Fig. 2. Total (A) and ^{15}N-labelled fertilizer (B) nitrogen distribution in grain proteins during grain development. (Legend: I: protein fraction extracted with 5% K_2SO_4, containing non-proteins, albumins and globulins; II: gliadin fraction extracted with 70% ethanol; III: other fractions. Values represent those of plants given 80 kg ha^{-1} N before sowing + 60 kg ha^{-1} labelled fertilizer N at Feekes stage 2–3 (treatment 2 in Table 1)

fertilizer nitrogen incorporated into the salt-soluble fraction decreased significantly, while that incorporated into the gliadin fraction rose. A similar scheme of N incorporation was described by several authors (Kapoor and Heiner, 1982; Borghi et al., 1983; Kaczkowski et al., 1988; Kieffer et al., 1988). It can thus be concluded that the incorporation of fertilizer nitrogen applied in spring into the protein fractions examined took place in accordance with a genetically determined order and quantitative proportions of the protein fractions.

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RELATIVE EFFICIENCY OF LINE × TESTER AND TRIPLE TEST CROSS DESIGNS FOR DETERMINING GENETIC ARCHITECTURE OF YIELD AND ITS CONTRIBUTING ATTRIBUTES IN ADZUKI BEAN (*VIGNA ANGULARIS* /WILLD/ OHWI AND OHASHI)

H. K. CHAUDHARY

DEPARTMENT OF PLANT BREEDING AND GENETICS, HPKV, PALAMPUR–176 062, INDIA

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The genetic information for a set of 22 lines of adzuki beans obtained from line × tester (L × T), triple test cross (TTC) and its modification (simplified triple test cross) was compared. Data were recorded and analysed for seed yield per plant, clusters per plant, pods per plant, pod length, seeds per pod and biological yield per plant. Epistasis was found to be an integral component of the genetic variation for all the traits as indicated by triple test cross analysis. Even in the presence of epistasis, similar genetic information was generated from both the designs, that is, in general, additive and non-additive genetic variances were important for all the traits except clusters per plant, for which only additive gene action was found to be important. Among the lines and testers HPAB 4 and HPU 51, respectively, were found to be good general combiners and L × T analysis showed HPAB 9 × A 1 to be a promising cross combination.

With regards to the relative efficiency of TTC and L × T, the former is advantageous in providing an unambiguous test for the presence of epistasis, while the latter provides additional information, particularly with regard to the gca and sca effects and variances, helping breeders in the choice of better parents. Hence, both these designs should be applied together in order to have concrete information about the genetic architecture of economic traits in any crop.

Key words: triple test cross (TTC), line × tester (L × T), *Vigna angularis*, adzuki bean, yield traits

Introduction

The experimental and analytical procedures of biometrical genetics are applied to study the genetic architecture of various populations. These procedures each have their own advantages and limitations and a method, good for a particular situation may not give similar valid genetic information under another situation. However, a biometrical procedure must have least assumptions and should provide more and reliable genetic information. A critical assumption about the absence of epistasis, which is known to be of wide occurrence in almost all the crop plants, must be included in any model for the estimation of gene action (Mather and Jinks, 1971). The line × tester analysis developed by Kempthorne (1957) provides information regarding gene action and sca effects along with their variances, yet takes care of the assumption about the absence of the independent action of non-allelic genes, which should otherwise be included in the model.

An extension of Design III of Comstock and Robinson (1952) called a triple test cross by Kearsey and Jinks (1968), and the modification (simplified triple test cross) introduced by Jinks et al. (1969) provide an unambiguous test of epistasis and make the coefficients of additive and dominance components equal, so as to have direct estimates of the degree of dominance from the additive and dominance variances. The simplified TTC makes use of 2n crosses, which is equivalent to L × T, but is able to provide more reliable information than the latter, particularly with regards to gene action. Hence, keeping in view the tremendous information generated from TTC and L × T designs with less effort, the present study was aimed at investigating the complete genetic architecture of different traits in a genetically unexploited crop, adzuki bean, and also to compare these designs with respect to their efficiency.

Materials and methods

The material for the present study comprised a set of 22 selected lines derived from the cross A 1 × HPU 51 of *Vigna angularis,* namely HPAB 1, HPAB 2, HPAB 3, HPAB 4, HPAB 5, HPAB 6, HPAB 9, HPAB 12, HPAB 15, HPAB 17, HPAB 18, HPAB 19, HPAB 20, HPAB 21, HPAB 22, HPAB 23, HPAB 25, HPAB 27, HPAB 31, HPAB 32, HPAB 35, HPAB 38 and 3 testers, namely A1 (L_1), HPU 51 (L_2) and their F_1, A 1 × HPU 51 (L_3). The 66 triple test cross progeny families (L_{1i}, L_{2i} and L_{3i}) and the 22 parental lines were grown in a single plant completely randomized design under rainfed conditions keeping a plant-to-plant distance of 30 cm. The material was scored for seed yield per plant (g), number of clusters per plant, number of pods per plant, pod length (cm), number of seeds per pod and biological yield per plant (g).

The variance of comparison ($\bar{L}_{1i} + \bar{L}_{2i} - 2\bar{L}_{3i}$) was used to test the presence of epistasis following Kearsey and Jinks (1968), where $\bar{L}_{1i,}$ $\bar{L}_{2i,}$ and $\bar{L}_{3i}$ are the mean values of the ith family in respect of the tester concerned. The sum of squares due to epistasis was partitioned into the Σ $[i]^2$ (additive × additive) type of interaction for 1 degree of freedom and the J (additive × dominance) and L (dominance × dominance) types of interactions for the remaining 21 degrees of freedom following Perkins and Jinks (1970). The mean squares due to the [i] type of interaction was tested as a variance ratio against the mean squares due to the J+L type of interactions whenever the latter were significantly greater than the appropriate within-family variances. An alternative test of epistasis based on the variance of ($\bar{L}_{1i}+\bar{L}_{2i}-\bar{P}_i$) was also employed, where $\bar{P}_i$ is the mean of the ith parent. The simultaneous application of these tests discriminates between the two causes of the failure of the simple additive-dominance model, namely epistasis and the inadequacy of the testers due to common genes (Jinks and Virk, 1977). The significance of the mean squares for these comparisons was tested using the χ^2 test against the appropriate within-family variances.

The variances of ($\bar{L}_{1i} + \bar{L}_{2i} + \bar{L}_{3i}$) and ($\bar{L}_{1i} - \bar{L}_{2i}$) were simultaneously computed for the detection and estimation of additive and dominance genetic components. Additive variance (D) = $4\sigma^2$ s and dominance variance = $4\sigma^2$s – $4\sigma^2$d, where σ^2s and σ^2d are the variances due to sums and differences. The average degree of dominance = $(H/D)^{1/2}$. The covariance of ($\bar{L}_{1i} + \bar{L}_{2i}$) and ($\bar{L}_{1i} - \bar{L}_{2i}$) was calculated for all values of i to show the direction of dominance (Jinks et al., 1969) . In the absence of epistasis and correlated gene distribution this covariance can be expected to be as follows:

Cov. sums/differences = –1/4 F, where the magnitude and sign of F determines the magnitude and direction of dominance. The 44 L_{1i} and L_{2i} families were analysed for combining ability following Kempthorne (1957).

Results and discussion

Triple test cross (TTC)

Significant mean squares due to Test I – $(\bar{L}_{1i} + \bar{L}_{2i} - 2\bar{L}_{3i})$ and Test II - $(\bar{L}_{1i} + \bar{L}_{2i} - \bar{P}_i)$ detected the existence of epistasis for all the traits studied (Table 1). The failure of the additive-dominance model in the present material cannot be attributed to the inadequacy of the testers. Hence epistasis appears to be an integral component of the genetic architecture of different traits in the present material. Further partitioning of the epistatic term revealed that [i] type epistasis was significant for the number of clusters per plant, number of pods per plant and pod length, while J+L type was significant for all the traits studied. This indicated that all the three types of non-allelic interactions, namely additive × additive [i], additive × dominance (J) and dominance × dominance (L) were prevalent.

The analysis of variance for sums (additive) and differences (dominance) were performed to work out additive and dominance components irrespective of the significance of epistasis in order to assess the relative magnitude of the two

Table 1
Analysis of variance for testing epistasis, additive and dominance components for different traits in adzuki bean

Source	df.	Seed yield/ plant	Clusters/ plant	Pods/ plant	Pod Length	Seeds/ pod	Biological yield/plant
Mean squares for epistasis							
Test I	22	8.16*	6.31*	33.38*	0.56*	0.83*	21.97*
Additive × additive [i] type	1	0.02	36.94*	158.84*	2.05*	0.51	25.48
Additive × dominance + Dominance × dominance (J + L) type	21	8.55*	4.85*	27.06*	0.49*	0.84*	21.80*
Error	590	4.57	2.67	15.56	0.16	0.35	12.15
Test II	21	6.56*	4.50*	26.16*	0.47*	0.71*	19.94*
Error	501	3.90	2.82	16.12	0.14	0.32	11.93
Mean squares for additive component	21	10.26*	5.43*	30.54*	0.78*	0.73*	28.50*
Error	590	4.57	2.67	15.56	0.16	0.35	12.15
Mean squares for dominance component	21	7.68*	4.95	37.30*	0.47*	0.70*	30.11*
Error	413	4.69	3.33	19.25	0.14	0.30	14.29

* $P < 0.05$

Table 2
Estimates of genetic components of variation for different traits following TTC and L × T analyses

Traits	TTC				L×T			Narrow sense heritability h^2
	Additive variance (D)	Dominance variance (H)	Average degree of dominance $(H/D)^{1/2}$	Cov. sums/ differences (F)	Additive variance (σ^2A)	Dominance variance (σ^2D)	Average degree of dominance $(2\sigma^2D/\sigma^2A)^{1/2}$	
Seed yield/plant	7.59	5.98	0.88	NS	5.86	3.00	1.01	43.24
Clusters/ plant	3.68	–	–	NS	8.63	–	–	–
Pods/plant	19.98	36.10	1.34	–518.21	47.44	18.05	0.87	55.98
Pod length	0.82	0.66	0.89	NS	0.96	0.33	0.82	67.13
Seed/pod	0.51	0.80	1.25	NS	0.12	0.40	2.58	15.00
Biological yield/plant	21.80	31.64	1.20	–654.17	20.76	15.82	1.23	40.81

NS Non-significant
– Not calculated

genetic components. Significant mean squares due to sums and differences for all the traits, except the number of clusters per plant, for which only mean squares due to sums was significant (Table 1), exhibited the presence of both additive and dominance variances for all the traits except clusters per plant, for which only the former was present. The average degree of dominance was >1 for the number of pods, number of seeds per pod and biological yield per plant, whereas it was <1 for seed yield and pod length (Table 2). Similar findings have been observed by Chaudhary et al. (1991, 1993a and 1993b) in adzuki bean.

Line × tester (L × T)

The analysis of variance for the combining ability of different traits, presented in Table 3, indicated significant differences due to general combining ability (GCA) among the lines for all the traits. The testers also differed with respect to GCA for all the traits except seed yield per plant. Significant mean squares due to lines × testers for all the traits except number of clusters per plant revealed that non-additive gene action was present for all the traits except number of clusters per plant. The average degree of dominance was greater than one for seed yield, number of seeds per pod and biological yield per plant and less than one for the rest of the traits (Table 2). Narrow sense heritability estimates for different traits indicated that heritability was high (>50 %) for pod length and number of pods per plant, moderate (30–50 %) for seed yield and biological yield per plant and low (<30 %) for number of seeds per pod (Table 2).

Table 3
Analysis of variance for combining ability of different traits in adzuki bean

Traits	Mean squares due to				
	Crosses 43	Lines 21	Testers 1	Lines × Testers 21	Error 413
Seed yield/plant	8.93*	10.61*	0.04	7.68*	4.69
Clusters/plant	7.95*	6.58*	99.93*	4.95	3.33
Pods/plant	49.35*	37.13*	559.21*	37.30*	19.25
Pod length	0.90*	0.85*	11.01*	0.47*	0.14
Seeds/pod	0.86*	0.98*	2.04*	0.70*	0.30
Biological yield/plant	37.05*	33.45*	258.46*	30.11*	14.29

* $P \leq 0.05$

TTC vs. L × T

The relative importance of additive and dominance components in the TTC analysis is in complete agreement with the results of L × T analysis for biological yield per plant, number of clusters per plant, pod length and number of seeds per pod. However, number of pods per plant showed a relatively greater importance of the dominance component in TTC and seed yield per plant in L × T analysis. This discrepancy in the relative importance of additive and dominance gene effects may be attributed to the biased estimates of the two components of genetic variation because of the presence of epistasis. However, even in the presence of epistasis the nature of the gene action for other traits in triple test cross analysis was in agreement with that of combining ability analysis, suggesting that triple test cross analysis provides a reliable estimate of additive and dominance components even if epistasis is present in the material under investigation. Similar conclusions have been drawn by Chahal and Singh (1974) in *Gossypium* and Pooni et al. (1978) in *Nicotiana.*

Regarding the efficiency of triple test cross analysis and combining ability analysis to extract information pertaining to the nature of gene action, it should be mentioned that combining ability analysis gives only a crude estimate of the nature of the genetic variances (additive and non-additive). On the other hand, triple test cross analysis not only provides an independent test of epistasis but, in the absence of epistasis, an independent and equally precise estimate of additive and dominance genetic components. The partitioning of epistasis in the TTC analysis can indicate what portion of the epistatic component is fixable. The [i] type (additive × additive) of epistasis is fixable and can therefore be exploited as well as the additive component. The relative proportion of [i] type epistasis to the (J) and (L) sub-components will help in choosing the breeding methods to be adopted for exploiting these sub-components.

By and large, epistasis formed an important part of the genetic structure of the material in the present study. Therefore, this component warrants its detection, estimation and consideration in the formulation of suitable breeding programmes. If the presence of epistasis is overlooked, as is the case when using combining ability analysis, by assuming the absence of epistasis, not only would information about the implications of epistasis be lost, but the estimates of the additive and dominance components of genetic variation would be biased, resulting in a faulty breeding procedure. Triple test cross analysis also provides additional information about the direction of dominance on the basis of a comparison of the results obtained through the analysis of differences and the correlation of sums and differences.

In the present investigation, the covariance of sums and differences (F) was non-significant for all the traits except number of pods and biological yield per plant, for which it was significantly negative (Table 2). This indicated ambidirectional dominance for seed yield, pod length and seeds per pod, whereas decreasing alleles were dominant more often than increasing alleles for number of pods and biological yield per plant.

Combining ability analysis provides extra information regarding the combining abilities (general and specific) of the parental lines and crosses. In the present investigation, the significant and positive values of GCA effects for different lines for different traits revealed that, among the lines, HPAB 4 was a good general combiner for seed yield and number of clusters per plant, HPAB 6 for number of clusters, number of pods and biological yield per plant, and HPAB 12 for number of pods, pod length and number of seeds per pod; among the testers, A 1 was a good general combiner for pod length and number of seeds per pod and HPU 51 for number of clusters, number of pods and biological yield per plant (Table 4).

The absence of association between *per se* performance and combining ability effects for most of the traits in the present study revealed the importance of studies on combining ability to select desirable parents (Table 5).

On the basis of significant and positive SCA effects, the cross combination HPAB 9 × A1 was found to be best for seed yield, number of pods and seeds per pod, HPAB 15 × A1 and HPAB 38 × A1 for pod length, and HPAB 25 × HPU 51 for biological yield per plant (Table 5). These crosses are expected to produce desirable transgressive segregants as most of them involve one good and one poor or average combiner.

On an overall basis, the cross combination HPAB 9 × A1 was found to be the best for seed yield and a few of its contributing traits, as no epistatic effect was shown by the line HPAB 9. This cross combination is thus easy to handle. Both additive and dominance genetic components were present for these traits, with the predominance of the additive component for seed yield. Breeding procedures such as the pedigree breeding method can therefore be employed in this combination, though selection needs to be deferred to later generations.

Table 4
Estimates of general combining ability (gca) effects for different traits in adzuki bean

Lines	Seed yield/ plant	Clusters/ plant	Pods/ plant	Pod length	Seeds/ pod	Biological yield/plant
HPAB 1	-0.21	–0.57	–0.51	0.04	0.19	–2.34
HPAB 2	–3.33*	–1.89	–5.90	–1.12*	–0.88*	–5.20
HPAB 3	–1.72	–1.52	–2.99	0.23	–0.07	–2.78
HPAB 4	4.27*	2.64*	6.00	0.24	0.42	4.60
HPAB 5	–1.24	–1.14	–3.27	–0.05	0.19	–3.79
HPAB 6	1.57	4.10*	7.33*	0.39	0.23	6.46*
HPAB 9	–0.62	0.08	0.58	0.01	–0.29	–1.16
HPAB 12	2.21	2.34	6.44*	0.53*	0.84*	3.91
HPAB 15	–4.13*	–3.44*	-8.76*	–0.95*	–0.76*	–7.03*
HPAB 17	0.59	0.06	0.61	0.49	0.50	0.01
HPAB 18	1.87	0.83	3.26	0.46	0.57	3.56
HPAB 19	0.34	–0.22	0.14	–0.01	0.08	2.03
HPAB 20	–4.02*	–1.62	–4.80	–1.12*	–1.13*	–6.98*
HPAB 21	2.05	2.61*	5.09	0.50	0.57	3.12
HPAB 22	2.16	0.13	0.28	0.89*	1.06*	1.78
HPAB 23	2.51	1.78	4.21	0.55*	0.42	4.54
HPAB 25	1.51	–0.41	1.62	–0.13	–0.12	4.74
HPAB 27	0.46	–0.67	0.36	–0.42	–1.05*	0.73
HPAB 31	–0.94	–1.01	–2.69	0.25	0.23	–1.47
HPAB 32	0.86	0.73	1.29	0.65*	0.51	2.45
HPAB 35	–0.59	–1.28	–3.01	0.15	0.21	–1.09
HPAB 38	–3.49*	–1.59	–5.29	–1.58*	–1.69*	–6.05*
$SE(g_i)\pm$	1.53	1.29	3.10	0.26	0.39	2.67
$SE(g_i–g_i)$	2.16	1.82	4.38	0.38	0.55	3.78
Testers						
HPU 51	–	1.51*	3.56*	–0.50*	–0.22*	2.42*
A 1	–	–1.51*	–3.56*	0.50*	0.22*	–2.42*
$SE(g_i)\pm$	–	0.39	0.93	0.08	0.11	0.80
$SE(g_i–g_j)$	–	0.55	1.32	0.11	0.16	0.14

* $P \leq 0.05$
– not estimated

Although combining ability analysis does not provide an exact picture of the nature of gene action, its application along with TTC analysis will be of great help in framing a comprehensive genetic architecture of the material under investigation.

Table 5

Estimates of specific combining ability (sca) effects of promising crosses along with gca status of their parents and correlation of gca with *per se* performance for different traits in adzuki bean

Traits	Crosses	sca effects	gca status of parents		Correlation of gca with *per se* performance
			P_1	P_2	
Seed yield/plant	HPAB 9 × A1	4.28*	Average	Average	0.28
Clusters/plant	–	–	–	–	0.16
Pods/plant	HPAB 9 × A1	18.71*	Average	Poor	0.05
Pod length	HPAB 15 × A1	1.21*	Poor	Good	0.50*
	HPAB 38 × A1	0.88*	Poor	Good	0.50*
Seeds/pod	HPAB 9 × A1	1.18*	Average	Good	0.26
	HPAB 15 × A1	1.16*	Poor	Good	
Biological yield/ plant	HPAB 25 × HPU 51	7.48*	Average	Good	0.38

* $P \leq 0.05$

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PREBREEDING OF EARLY MATURING WHEAT GERMPLASM FOR A CONTINENTAL CLIMATE

L. LÁNG and Z. BEDŐ

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, MARTONVÁSÁR, HUNGARY

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After preliminary tests on material from the Martonvásár Cereal Gene Bank, 16 early genotypes of different origins were analysed in detail for agronomic traits. The loose correlation found between yield potential and heading and ripening dates for the genotypes tested (r = 0.651* and r = 0.607, respectively) proves that it is possible to reconcile earliness and productivity. Thanks to breeding progress, early forms with far better frost resistance than previously are now available. Far Eastern genotypes mature 2–4 days earlier than the earliest varieties of European origin, making them valuable as initial stock in the development of new breeding sources. High quality, winter-hardy wheats from Central and Eastern Europe can be used more easily in the efficient breeding of early wheat varieties adapted to a continental climate.

Key words: wheat, prebreeding, earliness, ripening, frost hardiness, breadmaking quality

Introduction

The majority of wheat varieties widely grown in Hungary are traditionally medium early varieties. Under Hungarian climatic conditions, which are characterised by extremely varied weather, often of a continental nature, these varieties are able in the long term to survive cold winters, frequently without snow cover, and come to maturity before the hot, dry summer really begins. The registered early maturing varieties have a vegetation period only 2–3 days shorter, and extra-early varieties are cultivated rarely, and then only temporarily.

The satisfaction of the demand for early varieties is complicated by a number of factors. Although the negative correlation between yield potential and earliness within a certain interval of the vegetation period (Coventry et al., 1993; Annicchiarico and Pecetti, 1993) does not necessarily prevent the development of high-yielding early varieties (Botezan et al., 1986), the number of such genotypes included in breeding programmes is nevertheless low. In a continental climate the critical factor in the development of early varieties is yield stability, especially the possession of adequate frost resistance and winter hardiness. The majority of earliness sources originate from low latitudes, where the winter hardiness of the genotypes is generally poorer than that required in Central Europe. On the other hand, there are very few extra-early genotypes among the more winter-hardy European wheats. Basic stock which combines satisfactory productivity, quality and yield stability is particularly rare. The breeding of the variety Martonvásári 18, registered in 1990, was a big step forward in this respect, since it can not only compete with later-maturing varieties as regards yield potential, but is also one of the best registered varieties for winter hardiness and for the majority of agronomic traits. A compromise had to be made in respect to quality, however, so even this variety cannot be regarded as an optimum starting point for breeding.

In order to select suitable basic breeding stocks, early genotypes of various origin from the Martonvásár Cereal Gene Bank were evaluated with respect to yield ability, agronomic traits and winter hardiness.

Materials and methods

Sixteen winter wheat genotypes of different origins were examined in the experiments (Table 1), with the extra-early maturing variety Martonvásári 18 (Mv 18), the early variety Martonvásári 19 (Mv 19) and the medium early variety GK Zombor as controls.

Table 1
Agronomic characters of early maturing winter wheats
Martonvásár, 1993

Variety/line	Origin	Days to heading	Days to ripening	Frost survival %	Yield t/ha
Mv 18 st.	Hungary	136	183	80	6.90
Mv 19 st.	Hungary	142	187	83	6.22
GK Zombor st.	Hungary	144	189	78	6.57
81142	China	130	177	66	5.28
Geurumil	S.Korea	128	<177	76	4.54
Prostor	Bulgaria	133	182	80	5.81
Kraka	Bulgaria	133	181	47	5.78
GT 251-83	Bulgaria	137	182	79	6.97
GT 2218-41	Bulgaria	139	181	68	6.91
GT 1518-4-3	Bulgaria	138	183	55	6.32
F29K4-22	Romania	133	178	82	6.22
F4141W1-1	Romania	138	186	87	7.16
F30K2-1	Romania	134	178	71	6.09
F132	Romania	136	182	75	6.15
SK 9551-1-88	Slovakia	136	181	67	5.78
SK 5871-7-87	Slovakia	139	181	76	6.35
Mironovskaya 29	Ukraine	134	181	86	6.57
Erythrospermum 1524	Ukraine	136	179	67	5.60
Erythrospermum 352	Ukraine	135	179	68	6.64
NS Rana 2 st.				16	
L.S.D. 5%				25	0.77

Yield potential was compared in an experiment set up in the experimental nursery of the Agricultural Research Institute of the Hungarian Academy of Sciences in 1992/93, on 8 m^2 plots arranged in a random complete block design with 4 replications. In order to evaluate earliness, records were made of the times of heading and ripening, expressed in terms of the number of days from January 1st. The maturity date was determined by analysing the moisture content of 3×3 ears every 2–3 days from June 24th onwards (20–34 days after heading); maturity was defined as the date when the ear moisture content dropped to 20% (Láng and Balla, 1987).

In order to characterise the quality of the varieties the thousand kernel weight (TKW), the wet gluten content (ICC Standard No. 137/1), the Brabender farinograph value and the farinograph quality category (ICC Standard No. 115/1) were measured.

The testing of frost resistance was carried out in the Martonvásár phytotron at –15°C according to the method of Veisz (1987). The variety NS Rana 2 was used as the frost-sensitive control.

Results and discussion

Following preliminary selection, 16 early-maturing genotypes were included in the experiment. Many early sources, particularly from the Far East, did not satisfy even the minimum criteria due to their extremely poor winter hardiness or other unfavourable characters, so these were not examined further.

An analysis of the heading dates of the varieties and lines indicates that the basic stock collected was earlier than the early maturing control variety Mv 19, and there were even types which headed earlier than the extra-early variety Mv 18 (Table 1). The Chinese and South Korean wheats examined headed 6–8 days earlier than Mv 18, while for the earliest European sources this figure was 2–3 days. A close significant correlation (r = 0.656***) could be demonstrated between the heading and maturing dates, in agreement with previous observations (Hänsel and Ehrendorfer, 1973; Peterson et al., 1985), so the heading date is a sufficiently accurate index for the estimation of the maturity period. In most cases there is no significant difference between the varieties in the duration of grain filling (Wiegand and Cuellar, 1981). In the case of late varieties the process may be somewhat shorter, as grain filling takes place under warmer conditions, but this has little influence on the heading-maturity correlation. There was a period of 42–49 days between heading and ripening, depending on the variety. The longest heading-ripening period was observed for early-heading varieties adapted to local climatic and growing conditions, where neither weather factors nor serious disease stress shortened the grain filling period. The variety Geurumil was a special case, as it matured within 40 days of heading.

Considerable differences could be observed between the genotypes as regards yield potential. The performance of the adaptable control varieties was equalled or almost equalled by several of the sources, which thus have good breeding value. The yield potential of the two earliest genotypes was substantially (18–29%) lower than that of the standard. This was due not so much to the vegetation period as to their susceptibility to diseases, since severe powdery mildew and stem rust infection considerably reduced their leaf areas. The low yield level was thus not surprising. For the genotypes tested, the correlation between yield potential and heading and ripening dates was not close ($r = 0.651^*$ and $r = 0.607$, respectively), so the development of early, high-yielding genotypes is not inconceivable.

With the exception of two varieties, the genotypes tested had survival rates of over 60% in the frost test, which simulates a cold winter without snow cover. This is an excellent result compared with the registered varieties and indicates that, provided the breeding stock or variety collection possesses a sufficiently wide range of genetic variability, it is possible to find extra-early genotypes with satisfactory or excellent winter hardiness for use as initial stock in further breeding. The extra early sources with good frost resistance are especially valuable, since the Mediterranean and Far Eastern basic stocks previously known and widely used were usually poorly winter-hardy winter wheats, or intermediate or spring wheats. The present studies indicate that exceptions can be found to the frequently demonstrated negative correlation between earliness and hardiness and that this correlation was not true of the genotype collection formed after preliminary selection. This is proved by the negligible correlations between heading date and frost resistance ($r = -0.036$) and between maturing date and frost resistance ($r = 0.044$). Due to the good frost resistance of the varieties and lines, this factor was not correlated to yield potential either ($r = 0.27$).

In recent years, the quality expected from wheat has risen considerably. This explains the fact that, while in previous years the poorer quality of early varieties did not prevent their cultivation, nowadays there is no market for a variety with poor quality even if it is extremely early and winter-hardy, with satisfactory agronomic traits. With regard to the quality parameters studied, the majority of the sources used in these experiments did not satisfy the criteria (Table 2). To be suitable for use as crossing partners they must have a farinograph value of A_2, or at least B_1, combined with a wet gluten content of at least 28%. The best quality was observed in Ukrainian, Romanian and Bulgarian varieties. The quality of the earliest maturing genotypes was poor or medium, making it difficult to use them in breeding.

When breeding for early varieties, two strategies are possible. Adaptable, early basic stocks with good winter hardiness, chiefly of European origin, can be used directly in breeding varieties intended for regions with a continental climate. When crossing extremely early sources from the Far East, very few productive progeny with good adaptability can be expected in the first crossing cycle due to the great differences in lodging and disease resistance and in quality, so a multi-cycled breeding programme is needed, in the course of which adapted lines are first developed for use in further crosses to breed competitive extra-early varieties. Despite the complexity of this task, it is worth continuing the development of basic breeding stock from these varieties in order to increase genetic variability and to reduce the vegetation period.

Table 2
Breadmaking quality of early maturing genotypes
Martonvásár, 1993

Variety/line	TKW	Gluten %	Farinograph value	Farinograph category
Mv 18 st.	48.0	27.3	50.0	B_2
Mv 19 st.	40.4	28.2	74.3	A_2
GK Zombor st.	38.6	26.6	51.1	B_2
81142	43.9	31.5	55.6	B_1
Geurumil	37.9	33.3	52.6	B_2
Prostor	44.5	25.3	53.7	B_2
Kraka	40.7	29.7	52.2	B_2
GT 251-83	45.7	26.3	72.2	A_2
GT 2218-41	41.5	29.6	48.6	B_2
GT 1518-4-3	44.2	29.0	65.9	B_1
F29K4-22	42.7	26.7	69.3	B_1
F4141W1-1	48.8	25.6	72.8	A_2
F30K2-1	45.0	33.1	70.2	A_2
F132	37.6	25.4	64.0	B_1
SK 9551-1-88	33.7	23.3	65.7	B_1
SK 5871-7-87	47.2	27.4	60.6	B_1
Mironovskaya 29	42.4	31.2	67.2	B_1
Erythrospermum 1524	30.7	25.2	71.3	A_2
Erythrospermum 352	39.9	27.4	71.6	A_2

TKW = thousand kernel weight

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Short communication

EFFECT OF TILLAGE SYSTEMS AND WEED MANAGEMENT PRACTICES IN TWO TRANSPLANTED LOWLAND RICE CROPS GROWN IN SEQUENCE

O. S. KANDASAMY and L. KRISHNAKUMAR

DEPARTMENT OF AGRONOMY, TAMIL NADU AGRICULTURAL UNIVERSITY, COIMBATORE, 641003, INDIA

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A field experiment was conducted to study the interactive effects of tillage systems with and without summer ploughing and weed management practices on the growth and yield of two rice crops grown in sequence during the dry and wet seasons. Summer ploughing (dry season) reduced the weed problem, especially the sedges, which are difficult to control with other methods. As a result of better weed control, puddling with tractor or power tiller enhanced rice productivity by 7–8%. In both seasons, puddling with power tiller or tractor cage wheel, supplemented with either hand weeding twice or application of butachlor 1.5 kg ha^{-1} + hand weeding, was found to be the most economic weed management method in a transplanted lowland rice-rice sequence.

Key words: tillage system, weed management, crop sequence, rice

Introduction

Rice fields can be colonised by aquatic, semi-aquatic and terrestrial plants depending on the cultivation methods and seasons (Moody and Droft, 1981). Weeds growing in association with the rice crop reduced the vegetative potential of the crop, which ultimately resulted in substantial yield losses (Kim et al., 1979). Effective weeding practices reduce weed growth and consequently increase the crop yield. Weeds vary in their growth habit and life cycle. Therefore, no single method gives continuous and effective weed control. This could be achieved throug direct methods (physical and chemical methods) used within systems of indirect methods such as land preparation, water control, planting method and fertility management (De Datta and Herdt, 1983). In a rice-rice cropping pattern, a reduction in weed growth in one crop should produce a carryover effect on the weeds and yield of the next crop (Navarez and Moody, 1989). The objectives of this study were to measure the interaction between tillage systems and weeding treatments on weed control and rice yield in a rice-rice cropping sequence.

Materials and methods

The experiment was conducted at the College Farm of the Agricultural College and Research Institute, Madurai, on a Madukkur series sandy clay loam soil during the 1991–92 dry and wet cropping seasons. The available nutrient status of the soil was characterised by low N, medium P and high K contents.

Two transplanted rice crops were grown in sequence. The experiment conducted in the first season (dry), to evaluate the effect of tillage systems, consisted of puddling methods (with tractor, power tiller or bose plough), either with or without summer ploughing, and weed management practices (hand weeding twice, butachlor 1.5 kg ha^{-1} + hand weeding or unweeded check) and was laid out in a split-split plot design with three replications. In the second season, being a wet season and the second rice crop in the sequence, the tillage systems (only the puddling methods) were applied across the same weeding treatments in a split-split-plot design.

The summer ploughing was done with a tractor-drawn cultivator (2 passes) during mid-May, utilising the summer rainfall. The puddling method was carried out in the respective sub-plots using tractor-drawn or power tiller-drawn cage wheel or bose plough. In plots receiving herbicide, the calculated quantity of butachlor (1.5 kg ha^{-1}) was mixed with sand and broadcast uniformly on the plots 3 days after transplanting (DAT). For both crops, a common dose of 100:50:50 kg N, P_2O_5 and K_2O ha^{-1} was applied. Twenty five day old seedlings were transplanted at a 15 × 10 cm spacing with two to four seedlings per hill.

Weed samples were taken from four 50 × 50 cm quadrats per plot outside the crop yield sample area at 60 DAT. Samples were composited, cleaned and separated into individual species, counted, oven-dried and weighed. Crop yield was determined from the net plot area and expressed in q/ha at 14% moisture.

Results and discussion

Effect of treatments on weeds

The major weed species present in the experimental field in both seasons were *Echinochloa colona, E. crus–galli* (grasses); *Cyperus rotundus, C. iria* (sedges) and *Trianthema portulacastrum* and *Portulaca oleraceae* (broad–leaved weeds – BLW). There was a variation in the weed density and relative density between the seasons. During the first season, the total weed density of 125 m^{-2} was dominated by sedges and broad leaved weeds with 40.8 and 38.4% relative density, while the remainder were grasses (20.8%). By contrast, in the second season grasses dominated (41.3%) and broad leaved weeds were reduced to 20.1% of the total population of 324 m^{-2}, while the sedges remained unaffected (38.6%).

Observations on weeds revealed that summer ploughing reduced the population and dry weight of weeds in the first season. The effect of summer ploughing in reducing the sedges, which are persistent weeds, difficult to control, was more pronounced. Due to summer ploughing, the vegetative propagules are exposed to drying sun, resulting in a lesser density of weeds (Moody and Mian, 1979). Grasses and BLW are chiefly propagated by seeds, which are fairly resistant to drying and remain dormant.

Tractor and power tiller puddling controlled most of the weeds, except BLW. The better puddling achieved with these two implements than with a bose plough, due to increased inversion and churning of the soil, may have led to the burial of seeds, resulting in a lower weed population and biomass. According to Majid et al. (1988), land preparation with rotary implements in wet conditions proved to be a superior technique among the tillage operations, as the weed stand was minimised (Table 1).

Table 1

Effect of treatment on weed density and dry weight at 60 DAT (log transformed) in a rice-rice cropping sequence

Treatment	First crop		Second crop	
	Weed density m^{-2}	Weed dry weight kg ha^{-1}	Weed density m^{-2}	Weed dry weight kg ha^{-1}
Summer ploughing				
No summer ploughing	1.02 (10.8)	2.35 (220.3)	–	–
Summer ploughing	0.95 (9.0)	2.25 (180.1)	–	–
$CD_{5\%}$	NS	0.04	–	–
Puddling methods				
Tractor puddling	0.96 (9.2)	2.28 (189.7)	1.32 (20.7)	2.33(212.9)
Power tiller puddling	0.97 (9.6)	2.28 (191.8)	1.33 (21.0)	2.35 (223.3)
Bose plough puddling	1.04 (10.7)	2.34 (215.6)	1.39 (24.3)	2.43 (271.0)
$CD_{5\%}$	0.06	0.03	0.04	0.05
Weed control				
Unweeded control	1.53 (35.5)	2.83 (674.6)	1.87 (76.2)	2.84 (715.0)
Hand weeded twice	0.69 (5.1)	2.01 (103.3)	1.07 (12.2)	2.10 (129.5)
Butachlor + HW	0.74 (5.7)	2.06 (11.45)	1.09 (12.6)	2.17 (150.0)
$CD_{5\%}$	0.09	0.07	0.06	0.03

Figures in parenthesis are original values

A large variation in weed population and dry weight was observed between the weed control treatments in both seasons. At 60 DAT, hand weeding twice and butachlor + hand weeding led to lower weed density and dry weight than in the unweeded plots. After the first hand weeding or pre-emergence herbicide application, the regrowth of weeds was effectively controlled by a second hand weeding at 35 DAT, resulting in a limited number of weeds (Singh and Singh, 1985).

Effect of treatments on yield and economics

The advantage of summer ploughing in the first season was evident from a significant increase in grain yield (10.1%) over no summer ploughing. This might be due to an improvement in the number of panicles m^{-2} (Table 2).

Table 2
Effect of treatments on yield and economics of a lowland rice-rice cropping sequence

Treatment	First crop			Second crop		
	Panicles m^{-2}	Grain yield q ha^{-1}	Benefit:cost ratio	Panicles m^{-2}	Grain yield q ha^{-1}	Benefit:cost ratio
Summer ploughing						
No summer ploughing	441.0	54.20	2.72	–	–	2.72
Summer ploughing	485.0	59.70	2.86	–	–	2.86
$CD_{5\%}$	17.7	1.98	–	–	–	–
Puddling methods						
Tractor puddling	475.0	58.30	2.89	417.0	54.6	2.75
Power tiller puddling	472.0	58.10	2.96	402.0	53.8	2.80
Bose plough puddling	441.0	54.40	2.54	380.0	50.1	2.40
$CD_{5\%}$	8.4	1.06	–	19.0	2.28	–
Weed control						
Unweeded control	384.0	46.80	2.61	385.0	44.5	2.58
Hand weeded twice	504.0	62.20	2.78	406.0	57.7	2.60
Butachlor + HW	500.0	61.80	2.99	408.0	56.2	2.77
$CD_{5\%}$	9.4	1.15	–	16.0	1.78	–

Various puddle characteristics influence the rice yield, as rice is an aquatic plant. In comparison with bose plough puddling, the present study shows that tractor and power tiller puddling increased the grain yields by 7.1 and 6.8% in the first season, and by 9.0 and 7.4% in the second season. A higher yield was produced as the result of an enhancement of growth and yield components due to reduced weed competition and increased uptake of nutrients (Majid et al., 1988).

Weed management in transplanted rice either by hand weeding twice or by the pre-emergence application of 1.5 kg ha^{-1} butachlor followed by hand weeding produced similar yields during both seasons. The increase in yields due to these treatments was 33 and 32%, respectively, in the first season and 29 and 26% in the second season over the unweeded control. Similar results were obtained by Devendra et al. (1986).

The analysis of the economic index (benefit : cost ratio) revealed that an integrated weed management practice consisting of summer ploughing followed by puddling either with a power tiller or a tractor, accompanied by hand weeding twice or pre–emergence herbicide (butachlor) followed by hand weeding, can maximise the yield and the benefit : cost ratio in two transplanted rice crops grown in sequence.

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Review

POTENTIAL ROLE OF GRASSLANDS IN SUSTAINABLE LAND USE

G. NAGY

DEPARTMENT OF RURAL RESOURCE MANAGEMENT, DEBRECEN AGRICULTURAL UNIVERSITY, DEBRECEN, HUNGARY

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Grassland is the most versatile crop on Earth and can be utilized by mankind in a great many ways. The choice of grassland use is determined by two main factors: ecological conditions and socio-economic considerations.

Until recent times the main exploitation of grasslands was to provide livestock with forage. However, there have been new developments in the main functions of grasslands, such as the provision and protection of soil and water resources, furnishing habitats for wildlife, and contributing to the attractiveness of the landscape. All these features are key elements in the potential role of grasslands in sustainable land use.

This paper aims to outline the most characteristic methods of grassland use, the elements of conservation in grassland use and the efforts made to meet agricultural and conservation objectives. It also gives an analysis of the present state of affairs in Hungary, ending with a set of conclusions which may help future developments in the country.

Key words: grassland, sustainable, grassland utilization, conservation objectives, agricultural objectives, wildlife habitat, emissions, species diversity

Introduction

Grassland is the second most vital resource for land use on the Earth, expressed in land area. Its area is more than twice as large as that of the arable land. Excluding Europe, its area exceeds the area of croplands on every continent. On a global scale, grasslands are the key to ruminant animal production. For centuries, the main target for ruminant animal production and grassland use has been to provide mankind with milk, meat and fibres. Social demands for these products are not diminishing and there are available export markets within Europe and all over the world.

Grasslands show the widest ecological amplitude among all the biomes of the Earth. In Europe, they are distributed roughly between isotherms –1°C and 18°C, being highly tolerant of extreme ecological elements such as frost, permanent excess, fluctuation or deficit of underground water, flooding in winter and drought in summer (Rychnovská et al., 1994). Grasses can colonize the most extreme soils (peat, marsh, saline, high pH, etc.) as well.

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Until the present time, in many countries and regions of Europe, the principles of grassland production and utilization have been focused on the cost-efficient production of highly digestible forage. The animal yields and outputs per unit area have been increased, but often with negative effects on landscape value and on the diversity of wild flora and fauna. However, because public opinion has changed, some of the modern grassland management techniques are now regarded as undesirable. Presently the task for grassland management is to develop new systems which lead to or conserve grasslands with a stable floristic composition, so that biodiversity is maintained or encouraged.

Examples from some countries have shown that it is possible to integrate the objectives of agriculture and conservation interests to a high degree, but policy support is necessary to ensure cost-effectiveness (Nösberger et al., 1994).

Grasslands as land use systems have the highest potential value in sustainable agriculture. As natural resources, grasslands play a very important role in the global control of the biosphere. Grasslands protect the soil, assist in soil formation, preserve natural water resources, and stabilize the gas components of the atmosphere with CO_2 fixation and O_2 emission. The grass biomass also serves to sustain soil conservation. The grass root system enriches the organic matter content of the soil, contributes to soil particle formation, and prevents soil erosion. The aboveground biomass provides protection against water and wind erosion (Láng, 1994; Nagy and Vinczeffy, 1993).

The term sustainability can be variously defined, as applied to the areas of grassland production and utilization. Sustainable grassland use can be managed in perpetuity, i.e. without lasting damage to the production system or the environment. In practical terms, this means that it must be economically sound and acceptable in relation to environmental requirements. In the case of intensive grassland production, one must ensure that unavoidable losses of nutrients must not exceed the limits set by society, and water, soil and air pollution must stay within acceptable levels (Mannetje, 1994). In the case of extensive grassland production, its management must be aimed at the restoration, conservation or development of the desired sward structure and composition, with high nature reserve value in the given area.

Over the last few decades, new social concerns have arisen, which affirm the essential role of grasslands in nature conservation. It has been proven that intensive animal and grassland production are associated with harmful emissions, caused by excess nutrients and the production of greenhouse gases, methane (CH_4) and nitrous oxide (N_2O).

Extensive production grasslands in some parts of Europe are simultaneously of great value for nature conservation, whilst semi-natural grasslands, next to forests, provide the main vegetation types in nature reserves. In industrialised countries, there is a move to convert areas of intensive production grassland into more diverse vegetation types, which is of special interest to nature conservation (Mannetje, 1994).

Grasslands are important landscape elements in many regions of Europe. Their main functions are: (1) to provide livestock with forage, (2) to provide and protect soil and water resources, (3) to furnish a habitat for wildlife, both flora and fauna, and (4) to contribute to the attractiveness of the landscape (Meer and Wedin, 1989).

The main expectations for a good quality meadow or pasture have considerably changed over the last century and have been influenced by socio-economic conditions to a high degree (Reich, 1907; Nösberger, 1993).

In the following, different possibilities for grassland use, the key elements of conservation, and the present Hungarian situation will be highlighted with respect to sustainable grassland production and utilization.

Types of grassland use

The different types of grassland use have been determined by two main factors: ecological conditions (soil, climate) and socio-economic conditions (habitat of population, agricultural policy, level of development, etc.). Grassland vegetation can be used for different purposes, for forage production, combined forage production and nature conservation, nature reserves and recreation (Mannetje, 1994). Categories for the different grassland systems can be set up from different points of view. As regards the level of intensity, high input, lower input (moderate level) and extensive grassland systems can be mentioned. A target-oriented view of grassland use employs the categories of foraging grasslands and special purpose grasslands.

High input grassland systems

These systems exist in certain developed countries in temperate regions, e.g. Western Europe. These systems are practised mostly on dairy farms. Farmers use high rates of N fertilizers and/or slurry, which may reach 400–500 kg ha^{-1}. They mostly graze the herbage produced, the rest being preserved as silage or hay.

When examined in term of sustainable agriculture, the system raises several questions. The main problem is that the overall efficiency of nitrogen, in terms of milk and meat production, is generally less than 20%. Under grazing conditions, a high percentage is returned to the soil as dung and urine. When the grass crop is harvested by cutting and conservation, the nitrogen is ultimately recycled through the slurry of winter-housed livestock.

Table 1

Mean nutrient surplus and conversion rates of 996 specialized Dutch dairy farms during the years 1983–86. (Conversion rate is defined here as nutrient output via animal products as a percentage of total nutrient input into the farm.)

	Soil	Plant nutrients		
		N	P	K
Surplus kg ha^{-1} yr^{-1}	Sand	487.0	32.0	125.0
	Clay	466.0	33.0	78.0
	Peat	462.0	30.0	94.0
Conversion rate %	Sand	14.5	32.9	13.9
	Clay	13.3	29.6	19.0
	Peat	13.5	31.5	16.7

Source: Aarts et al. (1988)

When high levels of fertilizer nitrogen (e.g. 400 kg N ha^{-1}) are applied to pastures on freely draining soils that are grazed by beef cattle, in the short term, 15–30% is lost by volatilisation as NH_3, particularly from urine, 5–10% by denitrification to gaseous NO_2 and N_2, and up to 40% by leaching of NO_3 into the groundwater. In the case of heavier land conditions, with impeded drainage, losses due to leaching are reduced, but denitrification losses increase (Garret et al., 1992).

Similar trends can be observed with other macroelements as well. The conversion rates of different elements, defined as nutrient output via animal products as a percentage of total nutrient input into the farms, have shown a relatively low conversion of applied nutrients (Aarts et al., 1988). The rates have been found to be about 13–14%, 29–33% and 13–19% on different soil types for N, P and K, respectively (Table 1). As a result there is a high annual surplus of N, P and K applications (Table 2) in high input grassland systems (Weissbach and Ernst, 1994).

The system can also be investigated according to the emission of greenhouse gases. Emissions include N_2O and CH_4 from biological resources within a grassland agriculture system (soil, rumen), CO_2 emissions from burning the fossil fuels used to drive farm machinery and to produce fertilizer, and other gas emissions from external sources that are associated with grassland systems (Mannetje, 1994).

A comparison of low and high intensity dairy farms in this respect (Bakken et al., 1994) has shown that the "global warming effect" of all emissions (CH_4, N_2O and CO_2) was 9.1 t $ha^{-1}year^{-1}$ CO_2 equivalent for a high intensity Danish dairy farm and 6.5 t $ha^{-1}year^{-1}$ CO_2 equivalent for a low intensity farm (Table 3).

Both nutrient budgets and gas emissions have to be given special attention when discussing the sustainability of high input grassland systems.

Table 2
Average annual nutrient balances in 1983–1986 for about 175 specialised dairy farms on sandy soils in The Netherlands

	Nutrient (kg $ha^{-1} year^{-1}$)		
	N	P	K
Inputs:			
artificial fertilizers	331	15	30
purchased concentrates	137	25	74
purchased roughage	44	6	34
atmospheric deposition	48	1	4
miscellaneous	8	1	4
Total	568	48	146
Outputs:			
milk[1]	67	12	19
sold livestock[2]	14	4	1
sold roughage	1	0	0
Total	82	16	20
Inputs-Outputs	486	32	126

[1]about 13000 kg/ha, [2]about 540 kg/ha, Source: Aarts et al. (1992)

Low input grassland systems

Low input grassland systems are associated with the use of clovers, mainly white clover, in seed mixtures for the renovation or establishment of productive grass swards. The reason for the use of clovers is that farmers can reduce N inputs on their grasslands, as clovers can fix remarkable quantities of N from the atmosphere into the soil. At a high technical level of production, this system is comparable to the high input systems and may produce similar yields of milk (Bax and Schils, 1993) or beef (Mould et al., 1993) per hectare. The weak points of the system are at present the lack of persistent cultivars (Ennik, 1982) and the unbalanced availability of fixed N during the vegetation period (Wilhelmy and Kornher, 1993). The main advantage of biologically fixed N is that it does not cost fossil fuel. Unfortunately the high rate of N fixed may result in excessive N emissions into the environment (Elgersma et al., 1993; Scholefield and Tyson 1992).

Extensive grassland systems

These systems prevail under unfavourable ecological conditions: high mountains, steep slopes, dry climatic or poor soil conditions, areas with a high water table, temporarily flooded wetlands, or areas where environmental

Table 3
The global warming effects of the various sources of gas emissions from grassland farming (expressed as t CO_2 equivalent year^{-1})

Gas type	HI farm		LI farm	
	ha^{-1}	total	ha^{-1}	total
N_2O	1.8	67	1.0	78
CH_4	5.0	185	4.1	319
Energy	2.3	85	1.4	109
Total	9.1	337	6.5	507

HI= high intensity, LI= low intensity, Source: Bakken et al. (1994)

regulations or measures do not make it possible to farm grasslands more intensively.

These systems are free from all the harmful effects that may threaten the sustainability of the environment. The grassland management is characterized by very low inputs, and farming is aimed at the utilization of grass biomes. There is no surplus nutrient budget and there are no extra gas emissions into the environment due to fertilization inputs.

Elements of conservation in grassland use

In a paper of this brevity it is not possible to present all the aspects that exist between the different factors and the species diversity of the different grasslands, so some of the more general tendencies which influence the conservation value of the swards will be examined.

Conservation value and ecological conditions

In general species-rich grasslands occur on sites where there is neither an excess nor a shortage of different ecological elements. The sward contains the highest variety of species when the water supply shows no surplus and nutrients are limited to some extent (Bakker, 1989; Nösberger and Charles, 1990; Berendse et al., 1992; Rychnovska, 1993). With an increase in the available water supply to the flooded wetland level, the number of species decreased from 82 to 11 (Rychnovska et al., 1985). A classification of the Hungarian grassland types (Table 4) also shows that the largest number of species can be found at a moderate level of available ecological conditions (soil, water supply).

Management and conservation value

The type and species diversity of a particular grassland is based only partly on ecological conditions. Human activities such as seeding, watering,

Table 4
Features of some frequent grassland types in Hungary

Number of evaluated grassland types	Level of water supply (%)			Average species number (extreme values)	Average quality ranking (min. 1; max. 5)	Average yield t ha^{-1}DM in natural stage
	good	moderate	poor			
36	36	35	39	42.4 (11–66)	2.2	1.5–2.5

After Vinczeffy (1993)

fertilizing, tilling, cutting and grazing, are decisive factors which determine the vegetation coverage of grasslands to the same, if not a higher degree.

The intensification of grassland management – as a general rule – results in a loss of floristic diversity (Green, 1990; Rychnovská et al., 1994). The level of intensity can be expressed in DM yield (Table 5), and it was found by Rychnovská et al. (1994) that an increase in DM production from 4–6 t DM ha^{-1} decreased the number of species in the sward.

On the basis of the above relationship, correlations have been established (Fig. 1) between the production-conservation and agricultural value of the grassland (Wilkins and Harvey, 1993). Not only plant species, but animal species have also shown a positive correlation with lower inputs of production (Fig. 2).

Table 5
Yield, number of species, and energy content of the hay for different permanent pasture associations

	Ryegrass pastures	Arrhenatherion (1)	Arrhenatherion (2)	Mesobromion (2),(3)
Intensity of management	very high	intermediate	low	very low
Number of species	20	30	40	60
Yield (t DM ha^{-1})	10–13	9–11	5–8	1–3.5
Energy lactation (MJ kg^{-1} DM)	6.0	5.2	4.9	4.6

(1) main species: perennial ryegrass, rough meadow-grass, dandelion, white clover; hay cut when the grass began to flower.
(2) mainly used for hay production; cutting at inflorescence emergence (intermediate intensity) or just after inflorescence emergence (low intensity).
(3) on dry and infertile soils; little or no fertilization; late hay cut.
Source: Jeangros and Schmid (1991)

Although extensive management generally has a favourable influence on the number of species in the sward, some kind of moderate management is necessary to maintain the desired sward composition, otherwise competitive species tend to overgrow the non-competitive ones (Blazková, 1989).

Sward structure and conservation value

Vegetation structure has a powerful influence on the diversity of the plants and animals in ecosystems. As a general rule, the greater the variety of plants in the vegetation, the greater the diversity of associated animals (Mitchley, 1993). Vegetation structure also has an influence on changes in sward composition, as plants respond to any changes in other species.

In sward structure, the height of the sward is an important element. A lower standing crop will lead to a more open sward structure and create niches for the immigration of new species. In this respect, the conservation value of the area surrounding a given site becomes very important (Nösberger et al., 1994).

On newly established grasslands, the number of species primarily reflects the composition of the seed mixture; these swards have a relatively lower conservation value, but this increases over time (Nielsen and Debosz, 1994).

Production inputs and conservation value

Of the production inputs, fertilization should to be mentioned first. Except in cases when the soil has an extremely low nutrient status, the lack of fertilization gives a higher species number in a sward (Nösberger et al., 1994);

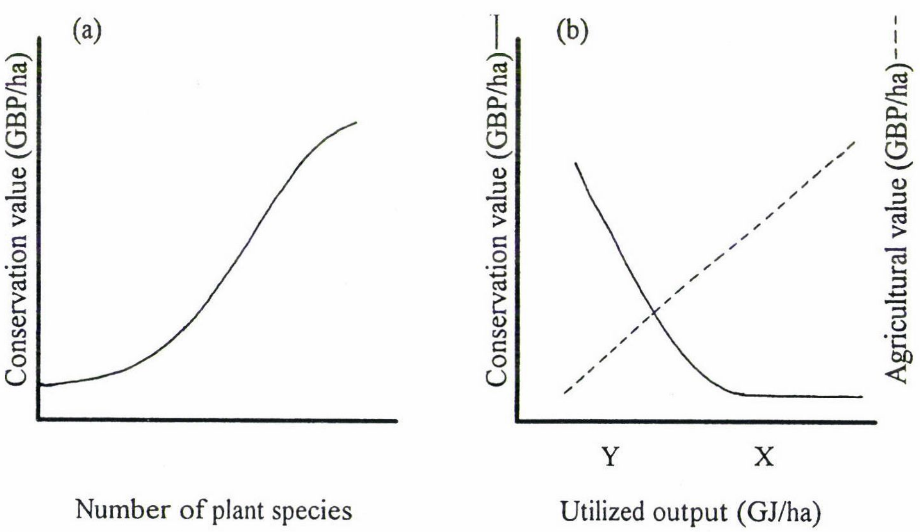

Fig. 1. Schematic relationships between number of plant species and nature conservation value (a) and between utilized output, agricultural value and nature conservation (b)
Source: Wilkins and Harvey (1993)

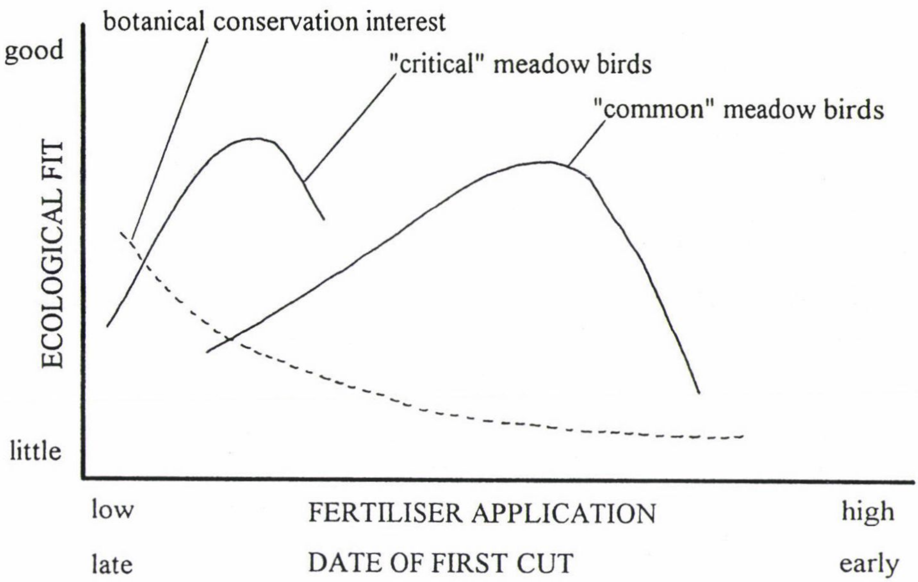

Fig. 2. Simplified relationships between the intensity of agricultural practices and the ecological fit for botanical and meadow bird conservation interest (Source: Dijkstra, 1991)

as a consequence, the use of fertilizers decreases the conservation value of the sward (Smith, 1993). As grasses in general respond well to nitrogen, N fertilization has the greatest effect in decreasing the number of legumes and herbs (Robotnov, 1993). N fertilization associated with other nutrients (P, K) or with intensive grazing may have detrimental effects on sward composition and conservation value (Meer and Wedin, 1989; Mountford et al., 1993). It has to be realized that the use of fertilizer may have a negative residual effect, leading to the species impoverishment of the sward (Tallowin et al., 1994; Rychnovská et al., 1994) even years after application.

At low soil nutrient content, a moderate level of fertilizers may increase the number of species in the sward. Manuring grassland with moderate rates of cattle slurry may be favourable to species diversity (Nösberger et al., 1994).

Ways of utilization and conservation value

Both grazing and cutting influence the sward composition. Controlled and traditional grazing (Papanastasis, 1994) helps to preserve species diversity. On pastures ungrazed for many years lack of grazing may result in a huge increase in rank grassland and the invasion of scrub (Toynton, 1993). In cutting utilization regimes, the date and number of cuttings have an effect on conservation value. The suggested date of cutting is always much later than the suggested grazing periods during primary growth (Nösberger et al., 1994, Bakker, 1989). The optimal cutting date is always in mid-July, any later cut is unfavourable for the number of species (Nielsen and Debosz, 1994). With the integration of grazing and cutting, late cutting and the grazing of the regrowth is suggested. With cutting only, a late first cut and a second autumn cut are advisable. Late cutting allows meadow birds to nest and hatch new generations to maintain their populations (Nösberger and Charles, 1990).

Restoration of the conservation value

Restoration is necessary after the intensive use of a grass sward or on abandoned croplands which once used to be (semi)natural grasslands. Possible methods of restoration are cultivation quite different from that practised so far (withdrawal of fertilizers, changing utilization regimes, etc.) or the reestablishment of the former sward. A certain amount of success has been achieved with wild flower and grass mixtures (Frame et al., 1993), but it has rarely proved possible to restore the original sward composition (Brown and Gibson, 1993).

Steps to meet agricultural and conservation objectives

If the up-dated objectives of modern grassland farming are to be met, a compromise between different interests will be required. To achieve this goal, a national strategy, political, social and economic support, and different sets of sophisticated projects are needed.

It is obvious that a national strategy has to be based on clarified surveys. A country has to know the value of her different grasslands, e.g. the National Vegetation Classification (Rodwell, 1992) described and classified the full range of British vegetation. The identification of Environmentally Sensitive Areas, and Sites of Specific Scientific Interest may help to develop national, regional and local projects (Baldock, 1993). Enthusiastic conservationists in different countries have started to work out the elements of a national conservation programme (Tishkov, 1994; Stypinski, 1994).

These programmes require a real compromise. It must be seen that nature conservation programmes will necessarily mean a lower output for farmers, whose losses must be compensated in some way. Different compensation programmes, called management agreements in some European Union Countries (Baldock, 1993), e.g. Britain, France, Ireland, The Netherlands, etc., are good examples of the possible compromises between farmers and environmental institutions (Mannetje, 1994). These national programmes are supported by international agreements, e.g. Reform of the Common Agricultural Policy, EC Agri-environment Regulation 2078/92 (Baldock, 1993). A compromise in the interests of grassland conservation and restoration can easily be achieved in well-developed countries, but it is more difficult in economically depressed countries. The overproduction of milk and beef can be solved by the extensification of grassland management in Western countries (Meer and

Wedin, 1989), but in the short-term opposite trends must be expected in Eastern Europe (Rychnovska, 1993).

Present Hungarian situation in grassland use

Production grasslands

There are about 1.2 million ha of grassland in the country, which comprises 21% of the total agricultural land. The average yield of the grassland is about 1.5 tons dry matter per ha, which – compared to other crops – is extremely low. This figure has not changed over the last 6–7 decades. On this basis grassland farming can be considered as extensive, which is due to both ecological and management reasons. The climatic conditions are not favourable for grassland production. The average daily temperature is 10°C for the year, and 16°C for the growing season. The winter is too hard and the summer is too hot for the grasses. The average annual rainfall (600 mm/year) is less than the optimum and its unpredictable distribution is quite frequently a real constraint for the growth of grass.

After the Second World War, the best quality grassland soils were ploughed up and turned into cropland. Grasslands have been left on marginal lands, which are not suitable for arable cropping. Fertility problems, low nutrient status (N), salinisation or high salt content, poor soil structure (extreme contents of sand or clay) and lack of water control can be listed among the unfavourable soil traits on the grasslands.

In Hungary, the inputs on grassland areas are extremely low. Fertilization, irrigation, improvement and renovation are all negligible. As a result of both poor climatic and soil conditions and low farming inputs, the grass yields are extremely low (1.5 t DM/ha), which leads to the occasional utilization of grasslands. According to professional estimations, only 1/3 of the grasslands are regularly utilized, 1/3 are sometimes utilized, while the last 1/3 are not utilized at all. In Hungary, grasslands are utilized for extensive animal production systems (breeding stock of sheep, dairy heifers, beef production). Dairy production is based on arable forage, corn silage and lucerne, which comprise about 800,000 ha of the total cropland (Nagy and Pető, 1995).

Grasslands for nature preservation

The role of grasslands in nature protection is less well-known, but is very important. Up to the end of 1994, 8% of the productive land had been declared as protected areas. The methods of land use on protected areas are shown in Table 6. It can be seen that, after forests, the establishment of grassland is the second most important land use on protected areas. However, on the most strictly protected areas (national parks, nature protected lands) the role of grassland is comparable to that of forests.

Table 6
Types of land use on nature protected areas (ha)

Protected areas	Cropland	Grassland	Forest	Others	Total
National parks	14.304	66.247	63.558	33.629	177.738
Landscape protected areas	68.362	94.567	252.592	51.132	466.653
Nature protected areas	2.451	7.526	11.028	5.225	26.230
Total *(ha)*	85.127	168.340	327.178	89.986	670.621
(%)	12.7	25.1	48.8	13.4	100.0

Changeable ecological conditions in the Carpathian Basin have created species-rich ecosystems. Many of these species have become endangered due to human activities (water control, production technologies, etc.). In the 1990s, 690 species were declared to be endangered, 500 species came under official protection, and 47 species were strictly protected (Nagy, 1994; Rakonczai, 1995). A large number of these species live on natural or semi–natural Hungarian grasslands (Nagy and Vinczeffy, 1993; Láng, 1995). It is in the national interest to preserve our natural heritage and to maintain the biological diversity of our grasslands.

Challenges for grassland use in the mid-90s

Over the last few years the ownership of the land, including grasslands, has been restructured. 46.2% of the total grassland is now owned by private farmers. Up till now nothing is known as regards the owners' plans for the use of their grasslands. Do they want to plough the grasslands up and use them as cropland? Unfortunately, there are examples of this. This would change the historical landscapes (wet meadows, sloping grasslands, etc.), and, as no permission is required to alter the grassland use, arbitrary cultivation may result in the permanent loss of sward compositions with conservation value (Nagy and Pető, 1995).

In the long term, the present agricultural policy plans to remove several hundred thousand hectares of arable land from cultivation and use them as grasslands or forests. The set-aside system is not known in Hungarian agriculture at present, but it can be predicted that agriculture will face this problem in the near future. In these programmes the multiple use of grasslands (for landscape design, erosion control, nature conservation) will have to play a very important role.

Conclusions

- The conservation value of grasslands in sustainable agriculture has been revealed over the last decades. The effects of different factors on the sward composition have been recognized. A thorough analysis of the present international situation may help the compilation of national, regional or local programmes aimed at the restoration, conservation or development of swards with high nature preservation value.

- Hungarian grasslands have avoided the problem of high inputs, so their conservation value is high; the most important threats to them are unexpected disturbance and the lack of utilization.
- It is necessary on a national scale to make a classification of our grasslands with their agricultural and conservation value, as also suggested by Márkus and Nagy (1995).
- As several hundred thousand hectares are expected to be removed from cultivation, their utilization has to be planned with regard to conservation aspects as well. Similar programmes have to be planned for the expected set-aside schemes.
- Nature restoration and conservation programmes cannot be successful without the support of farmers. Examples from some Western countries show that the reduction in output from farms participating in nature conservation programmes must be compensated through management agreements.

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Review

CLIMATIC PROGRAMMES USED IN THE MARTONVÁSÁR PHYTOTRON MOST FREQUENTLY IN RECENT YEARS

T. TISCHNER, B. KŐSZEGI and O. VEISZ

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, MARTONVÁSÁR, HUNGARY

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The climatic programmes employed in phytotron units are fundamentally determined by the research aims and strategy. However, when elaborating climatic programmes the technical parameters of the phytotron units must also be taken into consideration. In general the programmes simulate natural weather conditions, though special simplified programmes which do not reflect nature also exist. During the 25 years since the Martonvásár phytotron went into operation a large number of climatic programmes have been elaborated and utilised. Data will be presented here for those programmes which have been used most frequently for experiments in recent years. These include the T1, T3 and t2 programmes which simulate spring weather, and the Ny3 and ny2 programmes used to create summer conditions. Programmes M29, FDA and FDAS are based on autumn weather data and are used when testing the frost resistance of cereals. Special programmes have been elaborated for the raising of maize plants (Bk, Szk, Tk), sunflowers (Tsf) and tobacco (Tt).

Key words: climatic programme, phytotron, plant growth chamber

Introduction

Plant biologists have long been interested in how the major environmental factors required for the growth and development of experimental plants, such as temperature, light, air humidity, etc., could be created at any time of the year in a programmable, reproducible manner independently of the weather. By the middle of the century refrigeration, lighting and electronic regulation techniques had reached a standard which made it possible to manufacture plant growth equipment capable of satisfying these criteria. The plant research facilities containing these climatic units were named phytotron (from the Greek words phyto = plant and tron = house) at the suggestion of the head of the first phytotron built in Pasadena, USA in 1949 (Went, 1950).

The climatic programmes used in the plant growth equipment, or phytotron units, are fundamentally determined by the aims and strategy of the research. The research strategy in the Martonvásár phytotron, opened in 1972, was elaborated by Rajki (1973). This research strategy aimed to simulate natural conditions in the climatic programmes used for experiments. A method for calculating phytotronic climatic programmes by fitting trigonometric functions to climatic data series measured by meteorologists was elaborated in Martonvásár by Pletser (1973). Naturally, when preparing any specific climatic programme, the technical parameters of the given phytotron unit must also be taken into consideration. The major technical data of the plant growth units in the Martonvásár phytotron are presented in Table 1. With the exception of the vernalisation chamber and the gradient chamber, all the plant growth units in the Martonvásár phytotron were manufactured by the Canadian firm Controlled Environments Ltd. (Conviron).

In addition to the data in Table 1, other parameters, such as the precision of programming, the number of steps in which the lighting can be switched on and off, etc., must also be considered when preparing programmes. The climatic equipment installed prior to the reconstruction of the phytotron in 1990 was regulated on an analogous system, mostly using electromechanical switches (relays, timers, etc.). Depending on the type of phytotron unit, either 2 or 24 temperature values a day could be programmed, with a precision of 0.5°C. In all types the relative humidity could only be adjusted to two values (day and night).

Table 1
Technical parameters of the phytotron units

Phytotron unit			Growth		PPFD*	Temperature	
Designation	Type	No.	area (m^2)	height (m)	max. ($\mu mol/m^2s$)	min. (°C)	max. (°C)
Before reconstruction							
Autumn-winter chamber	PGV-36	12	3.3	2.4	500	–5	40
Spring-summer cabinet	E-15	12	1.4	1.8	500	5	40
Spring-summer cabinet	E-15VH	4	1.4	1.8	1000	5	40
Growth bench	GB-48	14	4.3	2.8	325	15	35
Cold room	C-812	2	7.1	0.5	–	–20	20
Gradient chamber	GRD-01	1	3.3	1.5	400	5	35
Germination cabinet	G-30	5	1.5	0.3	65	4	40
Vernalisation chamber	J-01	1	13.5	0.4	10	2	20
After reconstruction							
Autumn-winter chamber	PGV-36	12	3.3	1.9	600	–10	40
Spring-summer cabinet	PGR-15	16	1.4	1.8	600	4	45
Growth bench	PGB-96	6	9.0	2.6	500	10	40
Growth bench	GB-48	6	4.3	2.8	325	15	35
Cold room	C-812	2	10.8	0.5	–	–25	20
Gradient chamber	GRD-01	1	3.3	1.5	400	5	35
Germination cabinet	G-30	5	1.5	0.3	65	4	40
Vernalisation chamber	J-01	1	13.5	0.4	10	2	20
Tissue culture chamber	TCL	2	25.0	0.3	90	20	30

*Photosynthetic Photon Flux Density

The illumination system was based on Sylvania fluorescent tubes and incandescent lamps, which could be turned on and off in three steps. In each step a third of the lamps, and thus a third of the light energy, is switched on or off. Further technical details were reported by Tischner (1981). The plant growth equipment installed during the reconstruction have a digital regulation system using electronic switches (triacs). A total of 192 diurnal time lines can be programmed (discrete temperature, relative humidity, etc.), with a programming precision of 0.1°C or 1%. In a third of the units the light sources are fluorescent tubes and incandescent lamps, while the other two thirds use Tungsram daylight metal halide lamps. The illumination can be switched on and off in four steps. In each step a quarter of the lamps, and thus a quarter of the light energy, is switched on or off.

Although each new climatic equipment has its own programming and control unit, they are all linked to a central computer operating on a QNX operational system. Among other things, this computer is used to programme the phytotron units linked to the communication loop, to collect measurement data and to supervise the operation of the units. This means that the programme required for any experiment can be selected from the computer's climatic programme library at the press of a button.

Apart from the phytotronic climatic programmes which simulate natural climatic conditions, special, extremely simplified programmes are also used in many cases. In these programmes the simplification is frequently possible because of the nature of the experiment (e.g. the constant +2°C temperature and daily 8 h illumination at 1 klx in the vernalisation cabinet), while in other cases the simplification is necessary because of the very simple regulating system of the given phytotron unit.

In most cases, for financial reasons, the programmes are out of phase with the external time of day, so that the energy required for lighting can be supplied mostly at the cheaper night rate.

Climatic programmes

Using the method he elaborated, Pletser (1973) wrote more than twenty climatic programmes when research was first begun in the phytotron. These were based on meteorological data measured in Budapest between 1931 and 1950. Some of these programmes are still in use, despite the fact that the programming and regulating features of the new phytotron units installed during the reconstruction are far more advanced than those put into operation in 1972.

Among the climatic programmes elaborated during the first years of the Martonvásár phytotron, the most frequently used was the "T1" spring programme (the designation is taken from the Hungarian word "tavasz", meaning spring).

Table 2
The T1 climatic programme

Hours	Weeks								
	1	2	3	4	5	6	7	8	9
	Temperature (°C)								
1	6.5	8.0	9.0	10.0	11.0	12.0	13.5	14.0	15.0
2	6.5	8.0	9.0	10.0	11.0	12.0	13.0	14.0	14.5
3	6.0	7.0	8.5	9.5	10.5	12.0	13.0	13.5	14.0
4	5.5	6.5	8.0	9.5	10.5	12.0	13.0	13.5	14.0
5	5.5	6.5	8.0	9.0	10.5	11.5	12.0	13.5	14.0
6	5.5	6.5	8.0	9.0	10.5	11.5	12.5	13.5	14.5
7	6.0	7.5	8.5	9.5	12.0	13.0	14.0	14.5	15.0
8	6.5	8.5	10.0	11.5	13.0	14.5	16.0	17.0	17.5
9	9.0	10.5	11.5	13.5	15.0	16.5	17.5	18.5	19.5
10	9.5	11.0	13.0	14.5	16.0	17.5	18.5	19.5	20.5
11	11.0	12.5	14.0	15.5	17.0	18.5	20.0	21.0	22.0
12	11.5	13.5	15.0	16.5	17.5	19.0	20.0	21.0	22.0
13	12.0	14.0	16.0	17.5	19.0	20.5	21.0	22.5	23.0
14	12.5	14.5	16.0	17.5	19.0	20.0	21.0	22.0	23.0
15	12.5	15.0	16.0	18.0	19.0	20.0	21.0	22.0	23.0
16	12.5	14.5	16.0	17.5	19.0	20.0	21.0	22.0	23.0
17	12.0	13.0	15.0	16.0	17.5	18.5	20.0	21.0	22.0
18	11.0	13.0	14.5	16.0	17.5	18.5	19.5	20.5	21.0
19	10.0	12.0	13.0	14.5	16.0	17.5	18.5	19.0	20.0
20	9.0	10.5	12.0	13.5	15.0	16.0	17.0	18.0	19.0
21	7.5	9.0	11.0	12.5	14.0	15.0	16.0	16.5	17.5
22	7.5	9.0	10.5	12.0	13.0	14.0	15.0	16.0	17.0
23	7.0	8.5	10.0	11.5	12.0	13.0	15.0	15.5	16.0
24	6.5	8.0	9.5	11.0	11.0	12.0	14.0	15.0	15.0
	Relative humidity (%)								
Day	65	65	65	65	65	65	65	65	65
Night	75	75	75	75	75	75	75	75	75
Lights	Hours								
1/3 On	5.15	5.00	4.45	4.30	4.15	4.00	4.00	3.45	3.45
2/3 On	7.30	7.15	7.00	7.00	6.45	6.45	6.30	6.30	6.30
3/3 On	9.30	9.30	9.30	9.15	9.15	9.15	9.00	9.00	9.00
1/3 Off	14.00	14.00	14.15	14.15	14.15	14.15	14.15	14.15	14.30
2/3 Off	16.15	16.15	16.30	16.30	16.45	16.45	17.00	17.00	17.00
3/3 Off	18.30	18.45	18.45	19.00	19.15	19.15	19.30	19.30	19.30
	Illuminance (klx)								
3/3 On	40	40	40	40	40	40	40	40	40
	Photosynthetic Photon Flux Density (μmol/m^{2}s)								
3/3 On	500	500	500	500	500	500	500	500	500

Table 3
The M29 climatic programme

Hours	Weeks						
	1	2	3	4	5	6	7
				Temperature (°C)			
1	8.0	7.0	6.0	5.0	4.0	2.5	–3.0
2	7.5	6.5	5.5	5.0	3.5	2.0	–3.0
3	7.5	6.5	5.5	4.5	3.5	2.0	–3.0
4	7.5	6.5	5.5	4.5	3.5	2.0	–2.0
5	7.5	6.5	5.5	4.5	3.5	2.0	–2.0
6	7.0	6.0	5.5	4.5	3.0	1.5	–1.0
7	7.0	6.0	5.0	4.0	3.0	1.5	0.0
8	7.5	6.0	5.0	4.5	3.0	2.0	0.0
9	9.0	7.5	6.0	5.0	3.5	2.5	0.0
10	10.0	8.5	7.0	6.0	4.5	3.5	1.0
11	11.5	9.0	7.5	6.5	5.0	3.5	1.0
12	13.0	11.0	9.0	7.0	5.0	4.0	1.0
13	13.5	12.0	10.0	7.5	6.0	4.5	2.0
14	14.0	11.5	9.0	8.0	6.0	4.5	3.0
15	14.0	11.5	8.5	7.5	6.0	4.5	2.0
16	13.0	10.5	8.0	7.5	5.5	3.5	1.0
17	11.0	9.0	7.5	6.5	4.5	3.0	1.0
18	10.5	8.5	7.0	6.0	4.5	3.0	1.0
19	10.0	8.5	7.0	6.0	4.5	3.0	0.0
20	9.0	8.0	7.0	5.5	4.5	3.0	0.0
21	9.0	8.0	6.5	5.5	4.0	2.5	–1.0
22	9.0	8.0	6.5	5.0	4.0	2.5	–1.0
23	8.5	7.0	6.0	5.0	4.0	2.5	–2.0
24	8.5	7.0	6.0	5.0	4.0	2.5	–2.0
				Relative humidity (%)			
Day	70	75	75	*	*	*	*
Night	75	80	80	*	*	*	*
Lights				Hours			
1/3 On	6.15	6.15	6.45	7.00	7.00	7.15	3.00
2/3 On	8.00	8.00	8.15	8.30	8.45	8.45	3.00
3/3 On	9.45	9.45	9.45	10.00	10.00	10.00	3.00
1/3 Off	13.00	13.00	13.00	13.00	13.00	13.00	24.00
2/3 Off	14.45	14.45	14.30	14.30	14.30	14.30	24.00
3/3 Off	16.30	16.30	16.15	16.00	16.00	16.00	24.00
				Illuminance (klx)			
3/3 On	28	26	24	19	17	15	15
				Photosynthetic Photon Flux Density (μmol/m^{2}s)			
3/3 On	350	325	300	240	215	190	190

* At temperatures below 5°C the humidity is not regulated to avoid frost damage to the equipment

It was first used in genetic research carried out by Rajki (1982). The programme is based on the mean (50% frequency) Hungarian weather data for the period from the last week of March till the end of May, taking into account the fact that the lowest temperature which can be programmed in the E-15 growth cabinet is +5°C, and that 24 discrete temperatures can be programmed with a precision of 0.5°C. The technical parameters of the given phytotronic unit allowed the relative humidity to be programmed for day and night and the illumination to be switched on and off in three steps. The illumination intensity (which could only be measured in lux when the programme was prepared) was also restricted by the technical parameters of the unit, and the reduction in light flow as the light sources aged also had to be taken into account. The details of the "T1" programme are presented in Table 2. The programme has proved extremely useful particularly in the raising of vernalised cereals.

Another programme elaborated in the early days of the Martonvásár phytotron and still in use today in the testing of frost tolerance in cereals, as a preliminary growth programme simulating autumn weather conditions, is "M29" (Metodika 29). This was prepared taking into consideration the technical parameters of the PGV-36 plant growth chamber, in which the lowest temperature which can be programmed is –5°C (Table 1). The "M29" programme was elaborated for experiments on frost resistance testing carried out by E. Rajki (1980) and is still in use today (Veisz, 1993). The details of the programme are presented in Table 3. Although the programme was originally prepared for a lighting system consisting of Sylvania Cool White fluorescent tubes supplemented with incandescent lamps, it can be applied without modification in the new phytotron units equipped with Tungsram metal halide lamps (Tischner and Veisz, 1996). The second phase of hardening at –4°C and freezing at the temperature required in the experiment are carried out in units of the C-812 type. The three-week growth period following freezing involves a day/night temperature of 17/16°C at 75% relative humidity, with 14 h daylength and 125 $\mu mol/m^2s$ PPFD. Since the reconstruction in 1990 the basic "M29" programme can be modified to include changing values of atmospheric carbon dioxide concentration up to a maximum of 3000 μmol/mol (ppm) (Veisz and Tischner, 1995). This modification is particularly important in experiments concerning global climatic changes.

Sutka (1981) elaborated an autumn-type climatic programme (FDA) for studying the genetics of frost resistance in wheat, which is extremely simple and thus easily reproducible in other research institutes (Table 4). In the 6th week of the programme the hardening temperature can also be set to +2°C/–2°C. As in the M29 programme the second phase of hardening and freezing at the temperature required in the given experiment are carried out in a C-812 chamber. The conditions during further growth after freezing are also the same as in the M29 programme.

Table 4
The FDA climatic programme

	Weeks					
	1	2	3	4	5	6
			Temperature (°C)			
Day	15.0	10.0	10.0	5.0	5.0	2.0
Night	10.0	5.0	5.0	0.0	0.0	0.0
			Relative humidity (%)			
Day	70	75	75	*	*	*
Night	75	80	80	*	*	*
Lights			Hours			
1/3 On	6.00	6.00	6.00	8.00	8.00	2.00
2/3 On	6.00	6.00	6.00	8.00	8.00	2.00
3/3 On	6.00	6.00	6.00	8.00	8.00	2.00
1/3 Off	18.00	18.00	18.00	16.00	16.00	22.00
2/3 Off	18.00	18.00	18.00	16.00	16.00	22.00
3/3 Off	18.00	18.00	18.00	16.00	16.00	22.00
			Illuminance (klx)			
3/3 On	15**	15**	15**	15**	15**	15**
			Photosynthetic Photon Flux Density ($\mu mol/m^2s$)			
3/3 On	260	260	260	260	260	260

* At temperatures below 5°C the humidity is not regulated to avoid frost damage to the equipment

** With Gro-Lux/WS fluorescent lamps

An interesting point in this programme is that, since the only light source was Sylvania Gro-Lux/WS fluorescent tubes, the special spectral energy distribution of which is ideally suited to the spectral sensitivity of plant photobiological processes, the intensity of illumination was only 15 klx (Tischner, 1980).

In order to examine the dynamics of frost resistance in winter cereals, Veisz developed a completely different type of climatic programme. In this programme, designated "FDAS", the preliminary growth period prior to freezing is divided into two parts. During the first 10 days the plants are raised at day/night temperatures of 15°C/10°C with 12 h illumination, after which the temperature is reduced to a constant 2°C, again with 12 h illumination, and hardening is continued for any length of time required (generally 10, 20, 30... 100 days). The second phase of hardening, the freezing and the further growth after freezing are carried out as in the M29 programme. The capacity of the PGV-36 phytotron unit used for preliminary growth can be fully exploited,

Table 5
The T3 climatic programme

	Weeks					
	1	2	3	4	5	6*
	Temperature (°C)					
Day	16.0	16.0	17.0	17.0	18.0	18.0
Night	15.0	15.0	15.0	15.0	15.0	15.0
	Relative humidity (%)					
Day	65.0	65.0	65.0	65.0	65.0	65.0
Night	75.0	75.0	75.0	75.0	75.0	75.0
Lights	Hours					
1/3 On	5.15	5.15	4.45	4.45	4.15	4.15
2/3 On	7.30	7.30	7.00	7.00	6.45	6.45
3/3 On	9.30	9.30	9.30	9.30	9.15	9.15
1/3 Off	14.00	14.00	14.00	14.00	14.15	14.15
2/3 Off	16.15	16.15	16.30	16.30	16.45	16.45
3/3 Off	18.30	18.30	18.45	18.45	19.15	19.15
	Illuminance (klx)					
3/3 On	25	25	25	25	25	25
	Photosynthetic Photon Flux Density ($\mu mol/m^2s$)					
3/3 On	320	320	320	320	320	320

* For cereals, this programme is repeated until the end of flowering

because plants removed from time to time for freezing can be replaced by new plants at the start of the hardening period. At the end of the 100-day hardening period all the plants in the chamber are then frozen at the same time, which means that the experiment is also replicated (Veisz and Sutka, 1989).

In contrast to the climatic programmes which simulate natural conditions, the spring programme "T3" and the subsequent summer programme "Ny3" (from the Hungarian word "nyár", meaning summer) are based entirely on the limited technical features of the GB-48 growth bench (Tables 5 and 6). The GB phytotron unit can only be programmed for two (day and night) temperature values, and the lowest temperature which can be achieved, even when the illumination is switched off, is only 15°C. In the "T3" programme incandescent lamps cannot be used because they radiate heat. If necessary, the far-red range of the spectrum is provided by using Gro-Lux/WS fluorescent lamps. Experiments have shown a 1:1 ratio of Cool White and Gro-Lux/WS lamps to be optimum (Tischner, 1994). These climatic programmes have been used mainly in wheat breeding experiments (Balla, 1980).

Table 6
The Ny3 climatic programme

	Weeks					
	1	2	3	4	5	6
			Temperature (°C)			
Day	19.0	20.0	21.0	22.0	23.0	24.0
Night	15.0	15.0	16.0	17.0	17.0	18.0
			Relative humidity (%)			
Day	65.0	65.0	65.0	65.0	65.0	65.0
Night	75.0	75.0	75.0	75.0	75.0	75.0
Lights			Hours			
1/3 On	4.00	4.00	3.45	3.30	3.45	4.00
2/3 On	6.45	6.45	6.30	6.15	6.30	6.45
3/3 On	9.15	9.15	9.00	8.45	9.00	9.15
1/3 Off	14.30	14.30	14.30	14.30	14.30	14.30
2/3 Off	17.00	17.00	17.00	17.00	17.00	17.00
3/3 Off	19.30	19.30	19.30	19.30	19.30	19.30
			Illuminance (klx)			
3/3 On	25	25	25	25	25	25
			Photosynthetic Photon Flux Density (μmol/m^{2}s)			
3/3 On	320	320	320	320	320	320

In the course of the reconstruction in 1990, several GB units were replaced by PGB-96 chambers, the technical parameters of which (Table 1) made it possible to elaborate a new spring programme "t2" (Table 7), which is cooler than "T3" . This programme is followed by a new summer programme, "ny2" (Table 8). These programmes were first used in an experiment on drought tolerance (Farshadfar et al., 1995).

A modified, more dynamic version of "t2" is the "t21" programme (Table 9), followed by the summer programme "ny21" (Table 10). This programme has only recently been elaborated and is intended chiefly for the growth of spring cereals. In trial experiments it has produced very good results as regards both morphological traits and yield components.

The best example of the decisive role of the experimental aim when preparing climatic programmes is the phytotronic cultivation of maize plants. There is very little real difference between the "Tk" programme designed by Tischner, taking into account principally morphological aspects, and the "Bk" programme elaborated by Barnabás and Rajki (1976), who raised maize for pollen storage experiments. The "Szk" climatic programme created by Szundy for use in maize breeding, however, is of a quite different nature.

Table 7
The t2 climatic programme

Hours	Weeks									
	1	2	3	4	5	6	7	8	9	10
	Temperature (°C)									
00.00	10.0	10.0	11.0	11.0	12.0	12.0	13.0	13.0	14.0	14.0
00.30	10.0	10.0	11.0	11.0	12.0	12.0	13.0	13.0	15.0	15.0
01.00	10.0	10.0	11.0	11.0	12.0	12.0	14.0	14.0	16.0	16.0
01.30	10.0	10.0	11.0	11.0	13.0	13.0	15.0	15.0	17.0	17.0
02.00	10.0	10.0	12.0	12.0	14.0	14.0	16.0	16.0	18.0	18.0
02.30	10.0	11.0	13.0	13.0	15.0	15.0	17.0	17.0	18.0	18.0
03.00	10.5	12.0	14.0	14.0	16.0	16.0	17.0	17.0	18.0	18.0
03.30	11.0	13.0	15.0	15.0	16.0	16.0	17.0	17.0	18.0	18.0
04.00	11.5	14.0	15.0	15.0	16.0	16.0	17.0	17.0	18.0	18.0
04.30	12.0	14.0	15.0	15.0	16.0	16.0	17.0	17.0	18.0	18.0
14.30	11.5	14.0	15.0	15.0	16.0	16.0	17.0	17.0	18.0	18.0
15.00	11.0	13.0	15.0	15.0	16.0	16.0	17.0	17.0	18.0	18.0
15.30	10.5	12.0	14.0	14.0	15.0	15.0	16.0	16.0	17.0	17.0
16.00	10.0	11.0	13.0	13.0	14.0	14.0	15.0	15.0	16.0	16.0
16.30	10.0	10.0	12.0	12.0	13.0	13.0	14.0	14.0	15.0	15.0
17.00	10.0	10.0	11.0	11.0	12.0	12.0	13.0	13.0	14.0	14.0
	Relative humidity (%)									
00.00	76	76	76	76	76	76	76	76	76	76
00.30	76	76	76	76	76	76	76	76	73	73
01.00	76	76	76	76	76	76	73	73	70	70
01.30	76	76	76	76	73	73	70	70	67	67
02.00	76	76	74	74	70	70	67	67	64	64
02.30	76	74	72	72	67	67	64	64	64	64
03.00	74	72	70	70	64	64	64	64	64	64
03.30	72	70	68	68	64	64	64	64	64	64
04.00	70	68	68	68	64	64	64	64	64	64
04.30	68	68	68	68	64	64	64	64	64	64
14.30	70	68	68	68	64	64	64	64	64	64
15.00	72	70	68	68	64	64	64	64	64	64
15.30	74	72	70	70	67	67	67	67	67	67
16.00	76	74	72	72	70	70	70	70	70	70
16.30	76	76	74	74	73	73	73	73	73	73
17.00	76	76	76	76	76	76	76	76	76	76
Lights	Hours									
1/4 On	03.00	02.30	02.00	02.00	01.30	01.30	01.00	01.00	00.30	00.30
2/4 On	03.30	03.00	02.30	02.30	02.00	02.00	01.30	01.30	01.00	01.00
3/4 On	04.00	03.30	03.00	03.00	02.30	02.30	02.00	02.00	01.30	01.30
4/4 On	04.30	04.00	03.30	03.30	03.00	03.00	02.30	02.30	02.00	02.00
1/4 Off	14.30	15.00	15.30	15.30	15.30	15.30	15.30	15.30	15.30	15.30
2/4 Off	15.00	15.30	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00
3/4 Off	15.30	16.00	16.30	16.30	16.30	16.30	16.30	16.30	16.30	16.30
4/4 Off	16.00	16.30	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
	Photosynthetic Photon Flux Density (μmol/m^{2}s)									
4/4 On	200	200	250	250	270	270	320	320	350	350

Table 8
The ny2 climatic programme

Hours	Weeks					
	1	2	3	4	5	6
			Temperature (°C)			
00.00	15.0	16.0	17.0	18.0	19.0	20.0
00.30	16.0	17.0	18.0	18.0	19.0	20.0
01.00	17.0	18.0	19.0	19.0	20.0	21.0
01.30	18.0	19.0	20.0	20.0	21.0	22.0
02.00	19.0	20.0	21.0	21.0	22.0	23.0
02.30	19.0	20.0	21.0	22.0	23.0	24.0
15.00	18.0	19.0	20.0	21.0	22.0	23.0
15.30	17.0	18.0	19.0	20.0	21.0	22.0
16.00	16.0	17.0	18.0	19.0	20.0	21.0
16.30	15.0	16.0	17.0	18.0	19.0	20.0
			Relative humidity (%)			
00.00	76	76	76	76	76	76
00.30	73	73	73	76	76	76
01.00	70	70	70	73	73	73
01.30	67	67	67	70	70	70
02.00	64	64	64	67	67	67
02.30	64	64	64	64	64	64
15.00	67	67	67	67	67	67
15.30	70	70	70	70	70	70
16.00	73	73	73	73	73	73
16.30	76	76	76	76	76	76
Lights			Hours			
1/4 On	00.30	00.30	00.30	01.00	01.00	01.00
2/4 On	01.00	01.00	01.00	01.30	01.30	01.30
3/4 On	01.30	01.30	01.30	02.00	02.00	02.00
4/4 On	02.00	02.00	02.00	02.30	02.30	02.30
1/4 Off	15.00	15.00	15.00	15.00	15.00	15.00
2/4 Off	15.30	15.30	15.30	15.30	15.30	15.30
3/4 Off	16.00	16.00	16.00	16.00	16.00	16.00
4/4 Off	16.30	16.30	16.30	16.30	16.30	16.30
			Photosynthetic Photon Flux Density ($\mu mol/m^2s$)			
4/4 On	350	350	350	350	350	350

In this programme, in order to ensure a balance between organ differentiation and growth, there is a temporary drop in temperature after the appearance of secondary roots. Another characteristic feature of this programme is the relatively high temperature following pollination. The details of the climatic programmes used for raising maize plants are given in Tables 11, 12 and 13.

Table 9
The t21 climatic programme

Hours	Weeks					
	1–2	3–4	5–6	7	8	9
			Temperature (°C)			
00.00	10.5	11.0	11.5	12.0	13.0	13.0
01.00	10.0	10.5	11.0	11.5	12.0	12.0
02.00	10.0	10.5	11.0	11.5	12.5	13.0
03.00	10.5	11.0	11.5	12.0	13.0	14.0
04.00	11.0	11.5	12.0	12.5	13.5	15.0
05.00	11.5	12.0	12.5	13.0	14.0	16.0
09.00	12.0	12.5	13.0	14.0	15.0	17.0
12.00	12.5	13.0	13.5	15.0	16.0	18.0
14.00	12.0	12.5	13.5	15.0	16.0	18.0
15.00	11.5	12.0	13.0	14.0	15.0	17.0
16.00	11.0	11.5	12.5	13.0	14.0	16.0
17.00	11.0	11.5	12.0	12.5	13.5	15.0
20.00	10.5	11.0	11.5	12.0	13.0	14.0
			Relative humidity (%)			
00.00	74	74	74	74	74	75
01.00	75	75	75	75	75	75
02.00	75	75	75	75	75	75
03.00	74	74	74	74	74	74
04.00	73	73	73	73	73	73
05.00	72	72	72	72	72	72
09.00	71	71	71	71	71	71
12.00	70	70	70	70	70	70
14.00	71	71	70	70	70	70
15.00	72	72	71	71	71	71
16.00	73	73	72	72	72	72
17.00	73	73	73	73	73	73
20.00	74	74	74	74	74	74
Lights			Hours			
1/4 On	03.00	03.00	03.00	03.00	02.00	02.00
2/4 On	03.00	03.00	03.00	03.00	02.00	02.00
3/4 On	04.00	04.00	04.00	04.00	03.00	03.00
4/4 On	05.00	05.00	05.00	05.00	05.00	05.00
1/4 Off	14.00	14.00	15.00	15.00	15.00	15.00
2/4 Off	15.00	15.00	16.00	16.00	16.00	16.00
3/4 Off	16.00	16.00	17.00	17.00	17.00	17.00
4/4 Off	16.00	16.00	17.00	17.00	17.00	17.00
			Photosynthetic Photon Flux Density ($\mu mol/m^2s$)			
4/4 On	300	350	400	450	450	500

Table 10
The ny21 climatic programme

Hours	Weeks					
	1	2	3	4	5	6
			Temperature (°C)			
00.00	14.0	14.0	14.0	14.0	14.0	14.0
01.00	13.0	13.0	13.0	13.0	13.0	13.0
02.00	15.0	15.0	14.0	14.0	14.0	14.0
03.00	16.0	16.0	16.0	16.0	16.0	16.0
04.00	17.0	17.0	18.0	18.0	18.0	18.0
05.00	18.0	18.0	20.0	20.0	20.0	20.0
09.00	19.0	19.0	21.0	21.0	21.0	21.0
12.00	20.0	20.0	22.0	22.0	22.0	22.0
15.00	19.0	19.0	21.0	21.0	21.0	21.0
16.00	18.0	18.0	20.0	20.0	20.0	20.0
17.00	17.0	17.0	19.0	19.0	19.0	19.0
18.00	16.0	16.0	18.0	18.0	18.0	18.0
20.00	15.0	15.0	16.0	16.0	16.0	16.0
			Relative humidity (%)			
00.00	74	74	74	74	74	74
01.00	75	75	75	75	75	75
02.00	73	73	74	74	74	74
03.00	72	72	72	72	72	72
04.00	71	71	70	70	70	70
05.00	70	70	68	68	68	68
09.00	69	69	67	67	67	67
12.00	68	68	66	66	66	66
15.00	69	69	67	67	67	67
16.00	70	70	68	68	68	68
17.00	71	71	69	69	69	69
18.00	72	72	70	70	70	70
20.00	73	73	72	72	72	72
Lights			Hours			
1/4 On	02.00	02.00	02.00	02.00	02.00	02.00
2/4 On	02.00	02.00	02.00	02.00	02.00	02.00
3/4 On	03.00	03.00	03.00	03.00	03.00	03.00
4/4 On	04.00	04.00	04.00	04.00	04.00	04.00
1/4 Off	16.00	16.00	16.00	16.00	16.00	16.00
2/4 Off	17.00	17.00	17.00	17.00	17.00	17.00
3/4 Off	18.00	18.00	18.00	18.00	18.00	18.00
4/4 Off	18.00	18.00	18.00	18.00	18.00	18.00
			Photosynthetic Photon Flux Density (μmol/m^{2}s)			
4/4 On	500	500	500	500	500	500

Table 11
The Tk climatic programme

Hours	Weeks								
	1	2	3	4	5	6	7	8	9
					Temperature (°C)				
00.00	15.0	15.0	16.0	16.0	17.0	18.0	18.0	18.0	18.0
01.00	16.0	16.0	18.0	18.0	18.0	19.0	19.0	20.0	20.0
01.30	17.0	17.0	19.0	19.0	19.0	20.0	20.0	21.0	21.0
02.00	18.0	18.0	20.0	20.0	20.0	21.0	21.0	22.0	22.0
16.00	17.0	17.0	19.0	19.0	19.0	20.0	20.0	21.0	21.0
16.30	16.0	16.0	18.0	18.0	18.0	19.0	19.0	20.0	20.0
17.00	15.0	15.0	16.0	16.0	17.0	18.0	18.0	18.0	18.0
					Relative humidity (%)*				
Lights					Hours				
1/4 On	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2/4 On	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3/4 On	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
4/4 On	2.00.	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
1/4 Off	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00
2/4 Off	16.30	16.30	16.30	16.30	16.30	16.30	16.30	16.30	16.30
3/4 Off	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
4/4 Off	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
					Illuminance (klx)				
4/4 On	25	25	25	25	25	25	25	25	25
					Photosynthetic Photon Flux Density (μmol/m^{2}s)				
4/4 On	375	375	375	375	375	375	375	375	375

* The relative humidity is a constant 80% during the programme

When raising plants regenerated from tissue culture in the phytotron (Kovács et al., 1993) or interspecific hybrids produced by distant hybridisation (Molnár-Láng et al., 1991), which have poor vigour, the extremely simple "Mba" programme is used until the plants have established themselves. This involves a daytime temperature of 15°C with a relative humidity of 75% and a night temperature of 10°C with 80% relative humidity. The daylength is 16 h. The increase in temperature and illumination and the drop in humidity at the beginning of the day period takes place at 15-minute intervals, as does the reduction in temperature and illumination and the rise in humidity at the end of the day. The PPFD value is always determined by the maximum which can be achieved in the given phytotron unit (Table 1). Once the plants have established themselves, they are transferred to the T3 and Ny3 programmes, or other similar programmes.

Table 12
The Bk climatic programme

Hours	Weeks				
	1	2	3	4	5*
	Temperature (°C)				
00.00	16.0	16.0	18.0	18.0	20.0
00.30	17.0	17.0	19.0	19.0	21.0
01.00	18.0	18.0	20.0	20.0	22.0
15.00	17.0	17.0	19.0	19.0	21.0
15.30	16.0	16.0	18.0	18.0	20.0
16.00	15.0	15.0	17.0	17.0	18.0
	Relative humidity (%)**				
Lights	Hours				
1/4 On	00.00	00.00	00.00	00.00	00.00
2/4 On	00.00	00.00	00.00	00.00	00.00
3/4 On	00.30	00.30	00.30	00.30	00.30
4/4 On	01.00	01.00	01.00	01.00	01.00
1/4 Off	15.00	15.00	15.00	15.00	15.00
2/4 Off	15.30	15.30	15.30	15.30	15.30
3/4 Off	16.00	16.00	16.00	16.00	16.00
4/4 Off	16.00	16.00	16.00	16.00	16.00
	Illuminance (klx)				
4/4 On	25	25	25	25	25
	Photosynthetic Photon Flux Density ($\mu mol/m^2s$)				
4/4 On	375	375	375	375	375

* This programme is repeated until the end of the experiment
** The relative humidity is a constant 80%

In experiments set up for other institutes, individual climatic programmes are generally applied, depending on the test plant and the research aims, though it may happen that one of the routine programmes used in the Martonvásár phytotron is suitable. In some cases, for instance in experiments on sunflower and tobacco, programmes originally elaborated for individual cases have, over the years, become routine programmes. The details of the "Tsf" programme used for sunflowers and the "Tt" programme elaborated for tobacco are presented in Tables 14 and 15. In the case of tobacco, the seeds are sown in pots and then germinated at room temperature (21–23°C). The pots are not put into the phytotron unit under the given programme until the plants have emerged.

Table 13
The Szk climatic programme

Hours	Weeks											
	1	2	3	4	5	6	7	8	9	10	11	12
	Temperature (°C)											
00.00	15.0	17.0	15.0	11.0	14.0	14.0	16.0	17.0	15.0	17.0	18.0	20.0
04.00	16.0	18.0	16.0	12.0	16.0	16.0	18.0	19.0	17.0	19.0	20.0	22.0
04.30	17.0	19.0	17.0	14.0	18.0	18.0	20.0	21.0	19.0	22.0	23.0	25.0
05.00	18.0	20.0	18.0	16.0	20.0	20.0	22.0	23.0	21.0	24.0	25.0	27.0
05.30	19.0	21.0	19.0	18.0	21.0	22.0	24.0	25.0	24.0	26.0	27.0	29.0
10.00	19.0	21.0	19.0	19.0	21.0	24.0	24.0	26.0	26.0	27.0	28.0	30.0
14.00	19.0	21.0	19.0	18.0	21.0	22.0	24.0	26.0	24.0	26.0	27.0	29.0
18.30	18.0	20.0	18.0	16.0	20.0	20.0	22.0	23.0	21.0	24.0	25.0	27.0
19.00	17.0	19.0	17.0	14.0	18.0	18.0	20.0	21.0	19.0	22.0	23.0	25.0
19.30	16.0	18.0	16.0	12.0	16.0	16.0	18.0	19.0	17.0	19.0	20.0	22.0
20.00	15.0	17.0	15.0	11.0	14.0	14.0	16.0	17.0	15.0	17.0	18.0	20.0
	Relative humidity (%)											
00.00	80	80	80	80	80	80	80	80	80	80	80	50
04.00	79	79	79	79	79	79	79	79	79	79	79	50
04.30	78	78	78	78	78	78	78	78	78	78	78	50
05.00	77	77	77	77	77	77	77	77	77	77	77	50
05.30	76	76	76	76	76	76	76	76	76	76	76	50
10.00	76	76	76	76	76	76	76	76	76	76	76	50
14.00	76	76	76	76	76	76	76	76	76	76	76	50
18.30	77	77	77	77	77	77	77	77	77	77	77	50
19.00	78	78	78	78	78	78	78	78	78	78	78	50
19.30	79	79	79	79	79	79	79	79	79	79	79	50
20.00	80	80	80	80	80	80	80	80	80	80	80	50
Lights	Hours											
1/4 On	04.00	04.00	04.00	04.00	04.00	04.00	04.00	04.00	04.00	04.00	04.00	04.00
2/4 On	04.30	04.30	04.30	04.30	04.30	04.30	04.30	04.30	04.30	04.30	04.30	04.30
3/4 On	05.00	05.00	05.00	05.00	05.00	05.00	05.00	05.00	05.00	05.00	05.00	05.00
4/4 On	05.30	05.30	05.30	05.30	05.30	05.30	05.30	05.30	05.30	05.30	05.30	05.30
1/4 Off	18.30	18.30	18.30	18.30	18.30	18.30	18.30	18.30	18.30	18.30	18.30	18.30
2/4 Off	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00
3/4 Off	19.30	19.30	19.30	19.30	19.30	19.30	19.30	19.30	19.30	19.30	19.30	19.30
4/4 Off	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
	Photosynthetic Photon Flux Density (μmol/m^{2}s)											
4/4 On	250	250	270	270	300	300	350	350	400	400	450	450

Table 14
The Tsf climatic programme

Hours	Weeks				
	1–2	3-4	5*	6**	7***
			Temperature (°C)		
00.00	16.0	17.0	18.0	19.0	20.0
00.30	16.0	18.0	19.0	20.0	21.0
00.45	16.0	19.0	20.0	21.0	22.0
01.00	17.0	21.0	22.0	23.0	24.0
01.15	18.0	21.0	22.0	23.0	24.0
01.30	20.0	21.0	22.0	23.0	24.0
15.30	18.0	19.0	22.0	23.0	24.0
15.45	17.0	18.0	22.0	23.0	24.0
16.00	16.0	17.0	20.0	23.0	24.0
16.15	16.0	17.0	19.0	23.0	24.0
16.30	16.0	17.0	18.0	21.0	22.0
16.45	16.0	17.0	18.0	20.0	21.0
17.00	16.0	17.0	18.0	19.0	20.0
			Relative humidity (%)		
00.00	80	78	76	74	65
00.30	80	76	74	73	63
00.45	80	74	72	72	62
01.00	78	72	70	70	60
01.15	76	72	70	70	60
01.30	74	72	70	70	60
15.30	76	74	70	70	60
15.45	78	76	70	70	60
16.00	80	78	72	70	60
16.15	80	78	74	70	60
16.30	80	78	76	72	62
16.45	80	78	76	73	63
17.00	80	78	76	74	65
Lights			Hours		
1/4 On	01.00	00.30	00.30	00.30	00.30
2/4 On	01.00	00.30	00.30	00.30	00.30
3/4 On	01.15	00.45	00.45	00.45	00.45
4/4 On	01.30	01.00	01.00	01.00	01.00
1/4 Off	15.30	15.30	16.00	16.30	16.30
2/4 Off	15.45	15.45	16.15	16.45	16.45
3/4 Off	16.00	16.00	16.30	17.00	17.00
4/4 Off	16.00	16.00	16.30	17.00	17.00
			Photosynthetic Photon Flux Density ($\mu mol/m^2s$)		
4/4 On	300	350	400	450	500

*This programme is repeated until the beginning of flowering (generally for 4–6 weeks, depending on the variety)
**This programme is used during flowering (generally for 2–3 weeks)
***This programme is used during yield ripening, until the end of the experiment (approx. 5–7 weeks)

Table 15
The Tt climatic programme

Hours	Weeks				
	1–2	3-4	5*	6**	7***
			Temperature (°C)		
00.00	16.0	18.0	18.0	18.0	20.0
01.00	16.0	18.0	20.0	20.0	22.0
01.30	16.0	18.0	20.0	20.0	23.0
02.00	16.0	18.0	22.0	22.0	24.0
03.00	18.0	20.0	24.0	22.0	24.0
04.00	20.0	22.0	24.0	22.0	24.0
14.00	20.0	22.0	22.0	22.0	24.0
15.00	18.0	20.0	20.0	20.0	24.0
16.00	16.0	18.0	18.0	18.0	23.0
16.30	16.0	18.0	18.0	18.0	22.0
17.00	16.0	18.0	18.0	18.0	20.0
			Relative humidity (%)		
00.00	80	80	80	80	65
01.00	80	80	78	78	63
01.30	80	80	78	78	61
02.00	80	80	76	76	60
03.00	80	80	75	75	60
04.00	80	80	75	75	60
14.00	80	80	76	76	60
15.00	80	80	78	78	60
16.00	80	80	80	80	61
16.30	80	80	80	80	63
17.00	80	80	80	80	65
Lights			Hours		
1/4 On	03.00	03.00	01.00	01.00	01.00
2/4 On	03.00	03.00	01.00	01.00	01.00
3/4 On	04.00	04.00	02.00	02.00	01.30
4/4 On	04.00	04.00	03.00	03.00	02.00
1/4 Off	15.00	15.00	14.00	14.00	16.00
2/4 Off	15.00	15.00	15.00	15.00	16.30
3/4 Off	16.00	16.00	16.00	16.00	17.00
4/4 Off	16.00	16.00	16.00	16.00	17.00
			Photosynthetic Photon Flux Density ($\mu mol/m^2s$)		
4/4 On	300	300	400	450	500

*This programme is repeated until the beginning of flowering (generally for 7–8 weeks, depending on the variety)

**This programme is used during flowering (generally for 3–4 weeks)

***This programme is used during yield ripening, until the end of the experiment (approx. 4–5 weeks)

Epilogue

Over the last 25 years over three thousand experiments have been set up in the Martonvásár phytotron. The number of climatic programmes used for the experiments now approaches a hundred. Of these, however, only a dozen or so are still used regularly. Our aim in compiling this review of the detailed programmes was to facilitate the work of scientists when designing experiments and publishing the results. Table 16 provides a summary of the plant species included in the experiments, the research aims and the climatic programmes applied. Naturally, it is also our aim to provide detailed information of our climatic programmes for those working in other phytotrons or in laboratories equipped with plant growth equipment.

Table 16
Summary of the climatic programmes

Programme		Research aim
Code	Type	
T1	Spring	Raising of vernalised winter cereals
M29	Autumn-spring	Cereal frost resistance testing
FDA	Autumn-spring	Cereal frost resistance testing
FDAS	Autumn-spring	Cereal frost resistance testing
T3-Ny3	Spring-summer	Raising of vernalised cereals (e.g. for breeding)
t2-ny2	Spring-summer	Raising of cereals (e.g. for breeding)
t21-ny21	Spring-summer	Raising of spring cereals (e.g. for breeding)
Tk	Spring-summer	Raising of maize
Bk	Spring-summer	Raising of maize
Szk	Spring-summer	Raising of maize for breeding
Tsf	Spring-summer	Raising of sunflower for breeding
Tt	Spring-summer	Raising of tobacco for breeding
Mba	Spring	Raising of regenerants and interspecific hybrids

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Book reviews

JULIE MILLER JONES: Food Safety. Eagan Press, St. Paul, Minnesota, 1995. 453 pp. ISBN 0 962440736

Nowadays environmental protection is greatly concerned with water and air pollution, the disturbance of the ecological balance, the reduction in biodiversity, etc. In many cases these programmes pay little attention to nutrition as a risk factor, despite the fact that a constantly acting system is involved, containing both beneficial and harmful elements.

On the basis of twenty years of experience the author provides an excellent review of the effects, correlations and interactions of various fields. A systematic evaluation of toxic compounds of natural and artificial origin is given for each topic, stating whether these compounds are carcinogenic or mutagenic, or whether they induce histological abnormalities or teratogenic problems. A critical appraisal is given of the results of animal experiments; in some cases the author does not feel that the data are adequate to justify the banning of the compound in question.

The wide range of topics dealt with in the book can best be illustrated by listing the titles of the chapters: An overview of food safety; Regulating food safety; Establishing the safety of food components; Risk-benefit; Naturally occurring food toxicants; Bacteriological problems occurring in food; Molds and mycotoxins; Parasites, viruses, and toxins; How food processing affects nutritional quality and food safety; Food additives; Food colors and flavors; Food irradiation; Pesticides; Incidental contaminants in food; Radionuclides in food; Epilogue - where do we go from here?

A book review cannot give a detailed description of the material treated in the various chapters or discuss the various catastrophes mentioned. I would just like to quote one example: A woman in Minnesota died of cancer in 1977. Her daughter hoped that if she drank a lot of water she would wash the cancerous cells out of her body and would not die of cancer. The abnormal water intake led to renal shutdown, so she died from drinking water.

On a world scale nature protectionists are fighting for the elimination of pesticide residues and for a ban on food additives, not without some justification.

Natural nutrients contain the compounds essential for life: proteins, fats and carbohydrates, and other compounds which pass through the digestive system unchanged. They also contain compounds which provide protection against cancer. One group of compounds prevents the absorption of food, decomposes the nutrients and, what is worse, has a toxic, carcinogenic and teratogenic effect. These compounds are to be found in food in varying quantities and the body becomes accustomed to them. Paradoxically, we know less about the role of these compounds than we do about that of pesticides and additives.

A few examples of toxicity: in the USA potatoes contain 1–5 mg/100 g solanine, while in the UK this figure is 2.5–15 mg. The US standard authorises 20 mg/100 g. Potatoes which have turned green under the influence of light contain seven times this amount. Potato shoots have a teratogenic effect, while potato blight has been known to cause death. In the USA ripe tomatoes contain 36 mg/100 g tomatine. This ratio is far higher in green tomatoes. According to Dr Bruce, inventor of the mutagenity test, the ratio of pesticide residues to mutagenic compounds contained in food is 1:10,000.

Another group of compounds consists of the cyanides. It has long been known that the leaves and shoots of sweet sorghum contain 60-240 mg/g of these compounds. In black beans this ratio is 400 mg/100 g and in Pinto beans 17 mg/100 g. The quantities observed in apricot stones and almonds are 60 and 290 mg/100 g, respectively. These compounds inhibit respiration; the lethal dose of hydrogen cyanide is 30–250 mg for an adult male. In addition to numerous other toxic compounds, the bacteria, fungi, viruses and parasites consumed with food may have a similar effect.

The contradiction between risks and benefits is nowhere as great as in the case of pesticide application. Due to the careless use of pesticides, 100 people die annually in the USA and some 20,000 on a world scale. In some developing countries pesticides are also used as a means of committing suicide.

According to 1990 data, in the USA 13% of the yield is destroyed by pests, 12% by diseases and 12% by weeds. The damage caused by birds and mammals averages 1%, but in some cases may be as great as 50%. A total of 10,000 insect pests are known in the USA, of which some 200 cause significant damage each year; on a world scale this figure is in the range of 100,000. In Ethiopia one pest, the locust, destroyed 167,000 t of cereals, representing the food supplies of 1 million people, in a single year. Pathogens and pests destroy 47.3% of the rice, 23.9% of the wheat and 34.8% of the maize produced in the world. Consumers are prepared to accept the use of chemicals provided no residues remain. In many cases this is feasible.

The cost of treatment is low compared to the results; in the tropics the costs will be returned tens of times, while in European agriculture this ratio is less favourable. There tends to be a contradiction between consumer demands. For instance the apple varieties Red Delicious, Golden Delicious and McIntosh are popular, while no-one wants to buy Baldwin apples. However, the first three are susceptible to the fungal disease apple scab and need to be sprayed 7-10 times a year. Nevertheless, nobody is making any effort to popularise Baldwin.

The consumer raises somewhat schizophrenic demands – the produce should be free of disease, but no pesticides should be used. Plant breeding is unable to solve this task for all epidemics, not to mention weed control.

These few examples will perhaps serve to prove that the author has written an excellent book. All the conclusions are backed up by the analysis of a wide range of data. The publication of a Hungarian translation of this book would provide both consumers and agricultural experts with an extremely useful source of information.

A. BÁLINT

V. V. MORGUN, V. F. LOGVINENKO: Mutational Breeding of Wheat Plants. Naukova Dumka, Kiev, 1995. 627 pp. ISBN 5-12-004656-8

Wheat was one of the first plants ever to be cultivated. Nowadays its importance has by no means diminished; in fact, it is playing an ever increasing role in human food supplies. It may seem to an uninformed outsider that there is nothing new to say about this crop, but this opinion is refuted by the thick volume published in Russian (Mutatsionnaya selektsiya pshenitsi) by the authors.

From among the numerous interesting data given in the Introduction it is worth mentioning that wheat is grown on a total of 5 million hectares in the Ukraine. Approximately one tonne of wheat is produced per capita. According to the authors a period of some 13 years is required in the countries belonging to the Community of Independent States (CIS) for the production of a new variety. Each rouble spent on breeding brings a profit of 12.5 roubles.

In the first chapter, entitled "Development and principal trends of the investigations on genetic improvement of wheat", information is given on the various fields of research (crossing, selection, spontaneous and induced mutagenesis, germ plasm improvement, heterosis, biotechnological procedures, etc.). Attention is drawn to a very serious problem, that of the reduction in genetic variability. The varieties Mironovskaya 808 or Bezostaya 1 can be found in the geneology of 80–85% of the new varieties developed and registered in CIS countries, and many of them originate from direct crosses between these two varieties. The authors do not agree with those who consider that classical breeding methods (crossing, selection, mutational breeding) have been exhausted and will not lead to new results. They emphasise that the yield potential of varieties developed in this way is far from being fully exploited (36–65%).

Chapter 2, "Methods of obtaining and investigation of induced wheat mutations" discusses various mutagens and the identification of the mutants created.

Chapter 3, "Genetical activity of physical and chemical mutagenous factors", gives a full account of the genetic activity of mutagenic factors, the frequency and spectrum of mutations and the size of the dose.

Chapter 4, "Genetical activity of environmental factors", is a short chapter, divided into two parts. The first 20 or so pages provide an expert, precise account of the genetic consequences of the Chernobyl catastrophe. This is one of the most valuable parts of the book. To date over a 100 catastrophes have taken place involving radioactivity. One of the greatest of these was caused by the explosion in the Chernobyl reactor. In the Ukraine alone an area of 5.5 million hectares, inhabited by 2.5 million people, was contaminated. The book gives a survey of the multiplicity, degree and territorial extent of the damage and of its effect on living organisms, including the social and health aspects of the damage to human beings. The direction, form and extent of mutational changes caused by the irradiation is investigated in great detail at various distances from the site of the catastrophe. Extremely valuable observations were made on wheat. Many economically valuable mutants were found, with a high proportion of dwarf mutants. The results of research into the Chernobyl catastrophe were previously classed "Top secret". Many new discoveries and results in this field are published in this book for the first time. The second part of Chapter 4 deals with the mutagenic effect of the plant protection agents used by man.

Chapter 5, "Methods for increase of the rate and widening the spectrum of induced mutations", provides detailed information on the specific character of the action of mutagenic factors, the role of genotype in the mutational variability of wheat, the repeated and combined effects of mutagenic factors, the effects of mutagenic factors at different stages of ontogenetic plant development, and the coupling of combinative and mutation variability.

Chapter 6 discusses the "Use of experimental mutagenesis for solving specific problems of breeding".

Chapter 7, "Induced wheat mutants and their characteristics", reports on numerous mutations of biological and economic value (yield, quality, dwarfness, resistance). Wheat genes responsible for various traits and their chromosomal localisation are given on the basis of literary data.

In Chapter 8, "Use of mutants in wheat breeding", a detailed discussion is given of the possibilities and results of mutation breeding. While only 7 registered varieties were produced using this method between 1934 and 1954, a total of 1611 were created between 1985 and 1993, of which 1152 were selected directly from basic stock created by mutation, and 459 from hybrid populations in which one of the parental lines was a mutant.

At the end of the book there is an extremely brief summary in English, with a table of contents. An enormous number of literary data are cited. An understanding of the information published in the book is promoted by the numerous tables and figures. This monograph provides the reader with a far greater supply of knowledge than is suggested by its title. It would be well worth publishing it in English.

This substantial book can be recommended to breeders, biologists, environmental protectionists, researchers, university lecturers and post-graduates who wish to extend their professional knowledge.

L. SZUNICS and J. SUTKA

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Kiss, G., Papp, I., Bakondi-Zámori, E., Gartner-Bánfalvi, Á. (1977): A szója fungicides magcsávázásának és rhizóbium oltásának együttes tanulmányozása (Joint study of fungicide dressing and rhizobium inoculation in soybean). *Növénytermelés,* **26,** 147–153.

Ouyang, J. (1986): Induction of pollen plants in *Triticum aestivum.* In: Hu, M., Yang, M. (eds), *Haploids of higher plants **in vitro**.* Academic Press, Beijing, 26–41.

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CONTENTS

ORIGINAL PAPERS

Acta Agronomica Hungarica, Vol. 45(2), pp. 109 –116 (1997)

PLANT REGENERATION FROM SEEDLING APEX IN ANNUAL MEDICS

S. S. IBRAGIMOVA and S. E. SMOLENSKAYA

INSTITUTE OF CYTOLOGY AND GENETICS, SIBERIAN DEPARTMENT OF THE RUSSIAN ACADEMY OF SCIENCES, NOVOSIBIRSK, RUSSIA

(Received: 25 November, 1996; accepted: 25 March, 1997)

A four-step procedure was developed to induce plant regeneration via shoot formation in the annual medics *Medicago rigidula* (L.) Desr. and *Medicago orbicularis* (L.) All. Shoot buds originating from apex-derived calli of seedlings were preincubated on basal B_5 medium supplemented with 50 mM BAP for 10 days (M1 medium). Apex explants mainly from abnormal seedlings produced crumbly, green calli and shoot buds when cultured on medium containing 0.5 mM NAA and 5 mM BAP (M2 medium) for 3–4 weeks. To promote the development of shoot buds, calli were transferred to medium containing 0.1 mM NAA and 1 mM BAP (M3 medium). After 3–4 weeks, well-formed shoots were transferred to basal B_5 medium containing 5 mg/l IBA for rooting. All rooted plants of the two species were fertile.

Key words: annual medics, *Medicago rigidula*, *Medicago orbicularis*, callus induction, seedling apex, plant regeneration

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP:N^6-benzylaminopurine; IBA: indolyl-3-butyric acid; NAA: 1-naphthaleneacetic acid

Introduction

Regeneration capacity, as a biological character, is widely used in agricultural practice. However, plant regeneration as a character necessary in plant breeding has been inadequately studied. It is concluded from the literature that the regeneration capacity of plants is an important factor in breeding programmes. Regeneration reactions *in vivo* and *in vitro* have frequently been reported but very few of them, either in alfalfa or in other species, have been studied genetically (Reich and Bingham, 1980; Wan et al., 1988; Hernández-Fernández and Christie, 1989).

A new model for the genetic control of plant regeneration capacity is needed.

Methods based on the use of organ, tissue and cell cultures have been used in studies of plant regeneration. The *Medicago* genus, which includes perennial and annual species, proved to be advantageous in these studies. Considerable progress has been achieved in the development of tissue culture protocols for perennial alfalfa (Brown and Atanassov, 1985; Nagarajan et al., 1986). However, the complex genetic structure (autotetraploidy, allogamy, long life cycle) of perennial alfalfa poses limitations on basic and applied research in this species. In contrast, annual medics have genetic characters (diploidy, autogamy with high seed production per flower, short life cycle) which make them promising for genetic studies. Furthermore, the wild annual species of the *Medicago* genus are an important source of germplasm for the introgression of genes controlling insect and disease resistance into cultivated perennial alfalfa. It is documented that annual medics are difficult to regenerate. There are a number of reports dealing with the tissue culture of annual medics. Callus induction and plant regeneration have been obtained from leaf explants of *M. truncatula* Gaertn. (Nolan et al., 1989), hypocotyl-derived calli of *M. polymorpha* L. (Scarpa et al., 1993) and various tissue explants and protoplast-derived calli of *M. littoralis* Rhode. (Zafar et al., 1995).

However, the current *in vitro* techniques are unsatisfactory when applied to annual medic tissue cultures. Therefore, an efficient method must be developed for the plant regeneration of annual medics. Recent reports indicate that plants were regenerated when explants of *Phaseolus vulgaris* L. (McClean and Grafton, 1989), *Trifolium* species (Repkova, 1990) and *Phaseolus coccineus* L. (Angelini and Allavena, 1989) were cultured on a highly concentrated medium supplemented with BAP. The presence of BAP in the growth medium has been shown to promote the development of shoot buds or embryoids from intact seedlings in four *Phaseolus* species (Malik and Saxena, 1992). Phytohormones for the induction of plant regeneration were combined according to the protocols of Nolan et al. (1989) and Malik and Saxena (1992) in the present modification.

The major goal of this study was to determine the conditions optimal for plant regeneration from tissue culture and to select genotypes with high morphogenic capacity in *M. orbicularis* (L.) All. and *M. rigidula* (L.) Desr., pasture legumes grown in the southern regions of Russia (Khasanov, 1972).

Materials and methods

Seeds of annual medics *M. orbicularis* (L.) All. (No. 4842), *M. rigidula* (L.) Desr. (No. 2223) and *M. ciliaris* (L.) Krocker (No. 1496) were obtained from the Vavilov Institute of Plant Industry (St. Petersburg, Russia). The seeds were scarified with sandpaper and surface-sterilized in 96% ethanol (1 min) and 0.1% $HgCl_2$, followed by three rinses with sterile water. They were then placed on B_5 medium (Gamborg et al., 1968) supplemented with 30% sucrose, 1 g/l casein hydrolysate, 50 mM BAP and 8 g/l agar (M1 medium). The media were autoclaved at 1.5 atm for 20 min and the pH was adjusted to 5.8–5.9 prior to autoclaving.

The seeds were germinated in darkness at 24°C for 3–5 days and then transferred to 14 h photoperiods for 5–6 days. After 10–12 days of incubation, the seedlings were removed from the culture vessels and apex explants were prepared by removing the greater part of the cotyledons and cutting the stem and hypocotyl. The apex explants were placed on M2 medium containing 5 mM BAP and 0.5 mM NAA for callus induction. About 3–4 weeks after planting, the cultures were checked for the presence of callus and shoot buds. The calli were then transferred to M3 regeneration medium containing 1 mM BAP and 0.1 mM NAA. Regenerated plantlets were transferred to rooting medium containing 5 mg/l IBA. When the plantlets reached a height of 4–5 cm and had developed a root system, they were potted in a vermiculite-ceramsite mixture (3:1) and covered for 3–5 days with a glass cover to prevent desiccation. At a height of 10–12 cm the plantlets were transferred to pots containing a vermiculite-ceramsite mixture (1:3) to promote further growth in the greenhouse at 25°C.

The experiment was analysed in three replications. Intraspecific differences were estimated by Fisher's method.

Results and discussion

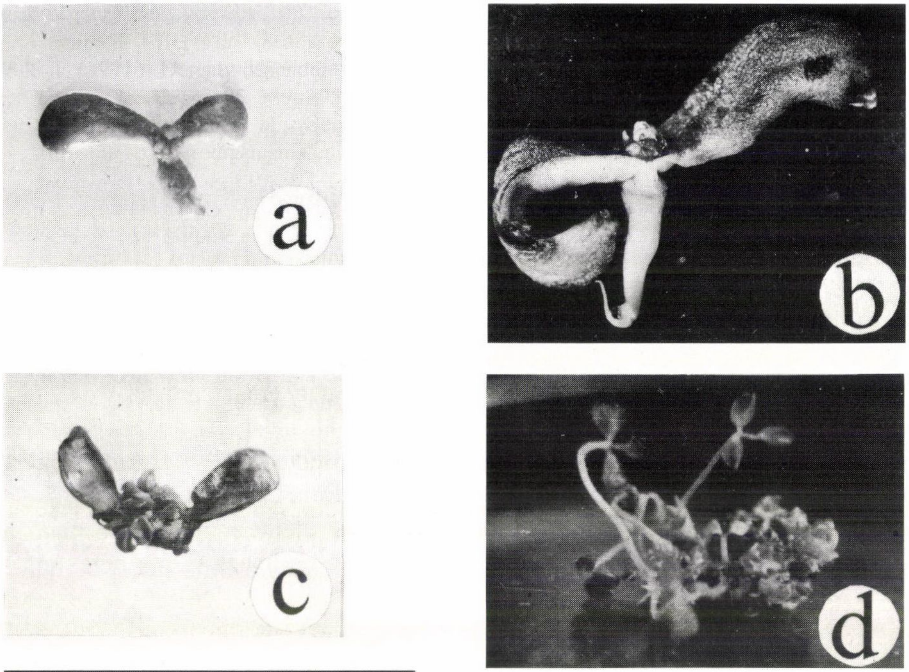
Calli were formed by cotyledon and hypocotyl segments of 7 day-old seedlings of *M. orbicularis, M. rigidula* and *M. ciliaris* on B_5 medium with different concentrations of 2,4-D and kinetin (5 mg/l 2,4-D : 1 mg/l kinetin; 2 : 2; 1 : 0.2). The callus growth rate and the frequency of callus induction were species-dependent. The induction frequency was highest in *M. rigidula* and *M. orbicularis,* where it reached 100%. No morphogenic response was obtained when the calli were transferred to hormone-free B_5 medium. The calli developed roots in all three species. The medium protocols elaborated for successful plant regeneration in perennial species are not suitable for annual medics.

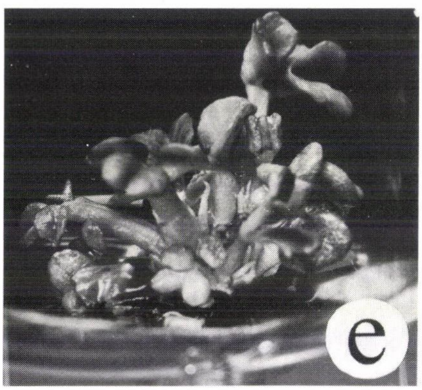
There are data indicating that BAP is one of the more active cytokinins. The application of BAP has a physiological effect on intact plants and stimulates seed germination (Kulaeva, 1973).

In the first study, seed of all the species germinated on B_5 medium containing 40, 60 and 80 mM BAP. The germination of the seeds of all three *Medicago* species was unaffected by the presence of BAP in the culture medium and occurred within a week. The frequency of seed germination varied between 70 and 100%. The high concentration of BAP affected callus induction, which was species-dependent. Callus formation was observed on the cotyledons, in the apex region or on the root. The transfer of cotyledonary or root-derived calli to regeneration medium did not stimulate plant regeneration. The calli induced in the apex region appeared more promising for further studies on plant regeneration.

M. orbicularis and *M. rigidula* were chosen for the analysis of regeneration. BAP, at a concentration of 50 mM, was found to be optimal for inducing regeneration in apex calli of annual medics. After 10–12 days of incubation, several seedlings developed a single shoot with cotyledons, one primary leaf and a root. Other seedlings formed large cotyledons, a reduced apex and reduced root. The first seedling group was designated as normal and the second as abnormal (Fig. 1a,b). Abnormal seedlings formed crumbly green callus in the apex region on BAP-supplemented basal B_5 medium (Fig. 1c). The number of cultured seedlings, the germination percentage of the seeds, abnormal seedlings, callus induction and the capacity for morphogenesis of both species is shown in Table 1.

Apices from normal and abnormal seedlings were transferred to M2 medium containing 5 mM BAP and 0.5 mM NAA for callus induction. The callus doubled in size after 10–15 days and became pale brown with green apices after incubation on M2 medium. To promote the further development of the





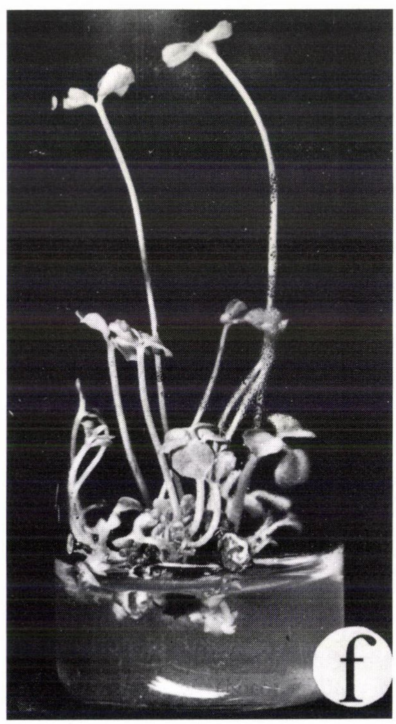


Fig. 1. Stages of regeneration in annual medics via shoot formation from seedling apex:
a-b: abnormal seedlings of *Medicago orbicularis* ×2 and *M. rigidula* ×2.5;
c: callus formation and shoot bud regeneration in the apex region of *M. rigidula* cultured on M2 medium ×2;
d: a shoot developing from callus of *M. orbicularis* cultured on M3 medium ×2
e-f: numerous shoots arising from apex-derived callus cultured on M3 medium: *M. orbicularis* (e –×4) and *M. rigidula* (f –×1.5)

Table 1
Regeneration of *Medicago rigidula* and *M. orbicularis* for callus culture

Characters	Medium	Species		Difference
		M. rigidula	*M. orbicularis*	
Number of seedlings		32	45	
Germination (%)	M1	100.0	100.0	
Abnormal seedlings (%)	M1	46.9±1.83	26.67±2.6	6.74*
Apex with callus induction (%)	M2	62.0±1.05	40.0±2.26	5.81*
Callus production of shoot buds (%)	M3	31.25±4.25	11.0±0.87	5.49*
Organogenic callus (%)	M3	21.87±2.1	11.0±0.87	
Embryonic callus (%)	M3	9.37±1.84	0.0	

*significant at P=0.05

shoot buds, the calli were transferred to basal M3 medium containing 1 mM BAP and 0.1 mM NAA. Gradually reduced concentrations of BAP and NAA in the culture medium lead to the development of shoot buds and multiple plantlet production.

All regenerants were obtained via organogenesis. The distinction between organogenesis and somatic embryogenesis was carried out on the basis of visual observation. Shoot formation was generally found on the callus surface. Embryo-like structures were observed in a previous experiment on the axial surface of the cotyledons and abnormal embryoids with cotyledon-like organs without roots on the callus surface in *M. rigidula,* but in neither case did they develop into normal plantlets on hormone-free medium.

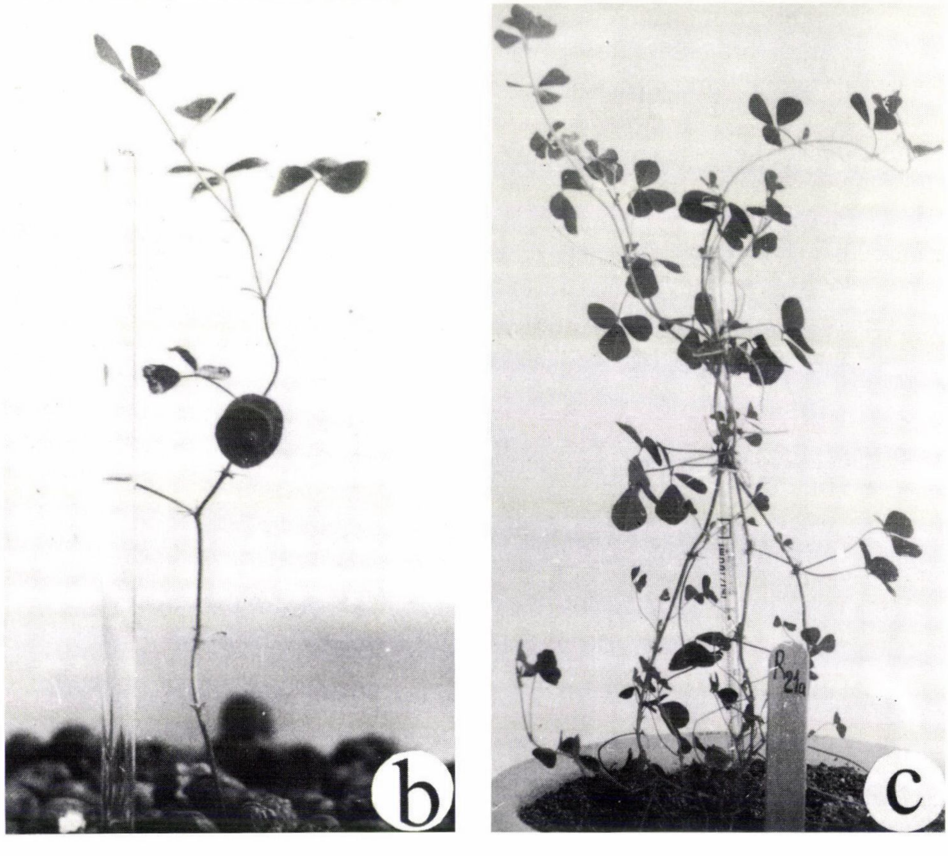
After two or three weeks of incubation, well-formed plantlets were transferred for rooting to medium containing 5 mg/l IBA (Fig. 2a). Several shoots rooted after 3–4 weeks. The others were incubated on the rooting medium for about 15–20 days, and formed roots later in pots with a vermiculite-ceramsite mixture. Shoot production from calli derived from seedling apices varied from one seedling and species to the other, and not all the shoots developed into plantlets (Table 2).

Table 2
Plant regeneration in cultures of seedling apices of *Medicago rigidula* and *M. orbicularis*

Lines		Number of shoots per seedling apex	Number of rooting shoots	Number of regenerants
M. rigidula	21a*	2	2	1
	22a	5	2	2
	14a	16	6	4
	16a	3	0	–
M. orbicularis	12a	5	2	1
	7a	4	1	1
	5a	2	0	–

Note : * apex from normal seedling

Fig. 2. Regenerated medic plants
a: a rooted plantlet of *Medicago rigidula* ×1.5;
b-c: regenerated plants grown in pots:
b: *M. orbicularis* ×1,
c: *M. rigidula* ×0.2 (vegetative stage)



Regenerated plants of both species grew to maturity, set seeds and had the same morphology as plants derived from seeds, but varied in height and pod production. Plants of *M. orbicularis* were very short and produced one or two pods per plant (Fig. 2b). Plants of *M. rigidula* varied in height and produced 3–25 pods per plant (Fig. 2c).

In most experiments callus was induced from parts of seedlings or adult plants (Nolan et al., 1989; Scarpa et al., 1993; Zafar et al., 1995), but cultivated intact seedlings have made it possible to identify regeneration capacity more accurately.

An interesting aspect of this study is the occurrence of normal and abnormal seedlings. Genotypes showing morphogenic capacity can be identified at this stage. The highest percentage of morphogenic calli and efficient plant regeneration were achieved from the apex-derived callus of abnormal seedlings (Table 1).

The procedure reported here may be generally applicable to other annual medics.

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GENETIC ANALYSIS OF THE FROST RESISTANCE OF WHEAT THROUGH DIALLEL ANALYSIS

O. VEISZ, Z. BEDŐ, L. LÁNG and T. TISCHNER

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, MARTONVÁSÁR, HUNGARY

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A twelve-parental diallel cross including reciprocals was carried out, and the F_1 hybrids and their winter wheat parents were used for a freezing test at –15°C under controlled conditions.

It could be concluded from the results of the diallel analysis that the dominance of frost resistance was not complete and that additive genetic variance was most important in the development of this property. The general reciprocal effects were not significant. With respect to frost resistance the variety Cheyenne had the best general combining ability among the varieties tested, followed by Martonvásári 4, Bezostaya 1 and Brule. When freezing was carried out at –15°C the dominant genes resulted in better frost resistance and the recessive genes in poorer resistance; in other words, frost resistance proved to be a dominant character.

Key words: *Triticum aestivum*, frost resistance, diallel analysis, growth chamber

Introduction

Most genetic studies involving winter hardiness in cereals have shown freezing resistance to be quantitatively inherited under the control of many genes (Eunus et al., 1962; Law and Jenkins, 1970; Shchipak, 1983). Knys and Norik (1971), Gullord (1974) and Gullord et al. (1975) were the first to apply F_1 diallel analysis to study the inheritance of frost resistance. Their results indicate that the development of this property is controlled by partially dominant genes. Puchkov and Zhirov (1978) tested the F_1 generation of a 7-parental diallel cross at –18°C and found that frost resistance was generally determined by an additive-dominant system, where the dominant genes tended to result in poorer frost resistance and the recessive genes in better resistance. In these analyses a significant reciprocal effect was not observed. In studies carried out by Beke and Sutka (1983) on the F_1 generation of a 6-parental diallel cross involving durum wheat varieties the general combining ability was found to be significant. The high GCA:SCA ratio suggested the prevalence of additive genetic variance. The general reciprocal effect was not significant in the reciprocal crosses.

Sutka (1981) tested the F_1 hybrids of a full diallel cross involving 6 winter wheat varieties and observed that the variance of both the general and specific combining ability was significant. The large GCA:SCA ratio indicated a preponderance of additive genetic variance. Covariance/variance regression analysis showed that frost sensitivity was partially dominant. The degree of dominance was less than 1, suggesting the incomplete dominance of frost sensitivity.

When studying the frost resistance and winter hardiness of winter × spring reciprocal triticale hybrids Shchipak (1983) and Shulyndin and Shchipak (1984) discovered individuals with frost resistance values between those of the parental forms in the F_1 generation. Reciprocal effects were observed, but the differences were not significant. Forms with good winter hardiness could be selected from the winter type plants. Orlyuk (1971) and Shelepov et al. (1980) were unable to find segregants surpassing the parents in crosses where the crossing partners included lines with good frost resistance. The lack of genetic gain in cold tolerance suggests a reduction in the genetic variability for this character (Fowler and Gusta, 1979; Grafius, 1981).

While some authors found freezing resistance to be the result of recessive genes with additive effects (Jenkins, 1969; Law and Jenkins, 1970), others reported both additive and non-additive effects, controlled by both recessive and dominant genes (Eunus et al., 1962; Rohde and Pulham, 1960). Amirshahi and Patterson (1956) found additive effects with no gene interaction.

The present paper describes experiments designed to study the combining ability and the inheritance of frost resistance in a twelve-parental diallel cross of winter wheat.

Materials and methods

The following 12 wheat varieties, with different degrees of frost resistance, were included in the full diallel cross: Bezostaya 1, Martonvásári 4, Martonvásári 8, Martonvásári 2068 (experimental), Martonvásári 10, GK Kincső, GK Szeged, Rivoli, Disponent, Brule, Bánkúti 1201 and Cheyenne.

The parents were raised under field conditions for the development of the F_1 generation. The crosses were carried out with the usual methods (castration, isolation and fertilisation). The frost resistance of the parents and the F_1 generation was tested under phytotronic conditions.

For the frost resistance test, germinated wheat seeds were planted. Germination was carried out in complete darkness at a day/night temperature of 20°/12°C with a 12-hour day. After 3 days of germination, seeds with intact rootlets were planted into a 4:1 mixture of earth and sand in wooden boxes with internal dimensions of 38×26×22 cm. The seeds were planted at a depth of 3.5–4 cm. Each box consisted of nine rows with 20 plants to a row.

The varieties were planted in four replications, with 20 plants per replication. After planting, the boxes were kept at room temperature for a day, after which they were transferred to an autumn-winter type growth chamber (Conviron PGV–36), where they were kept for 6 weeks. During this period the temperature, light intensity and length of illumination were gradually reduced with a weekly change of programme similar to autumn conditions in the field. Within each weekly programme, the temperature fluctuation, light intensity and daylength were the same each day. The daily temperature fluctuation followed the daily temperature changes experienced in nature. For details of the climatic programme, see Tischner et al. (1997).

The preliminary growth stage was followed by 2-phase hardening. The plants were exposed to the first phase in the autumn-winter chamber, where the temperature fluctuated daily between +3°C and –3°C with a 21-hour daylength and a photosynthetic photon flux density

(PPFD) of 190 μmol.$m^{-2}s^{-1}$. This phase lasted for one week. The second phase of hardening, which lasted for 4 days, took place in a frost resistance testing chamber (Conviron C–812) immediately prior to freezing. The temperature was a constant –4°C with no illumination.

Freezing was carried out in the frost resistance testing chamber set up in the phytotron specifically for this purpose. The temperature was gradually decreased. Freezing took place at –15°C for 24 hours, for both the varieties and the F_1 populations. Throughout the frost treatment the plants were kept in the freezing chamber at +0.5°C for 2 days.

After thawing the boxes were transferred to growth benches (Conviron GB–48). The plants were grown for a further 3 weeks at a day/night temperature of 17°/16°C, with a 14-hour daylength and a PPFD of 125 μmol.$m^{-2}s^{-1}$. At the end of the third week, plants which had survived freezing and had begun to develop were clearly distinguishable from those which had died. The result of freezing is given as a percentage of the plant number prior to freezing.

In the full diallel cross the evaluation of frost resistance as regards general (GCA) and specific (SCA) combining ability was carried out using the 1st method of Griffing (1956) and that of Keuls and Garretsen (1977). Hayman's (1954a, b) model was used for the estimation of the components of genetic variance and the method reported by Jinks (1954) for the graphic analysis of covariance/variance. The narrow and wide sense heritability index was estimated with the method elaborated by Mather and Jinks (1971).

Results

Single factor variance analysis was employed to determine the significant differences between the parents and progeny generations, the results of which are given in Table 1. It can be seen from the data that genotypic differences in frost resistance were significant at the 0.1 % level.

The mean values of frost resistance for the parents and the F_1 hybrids are presented in Table 2. Among the parents Cheyenne, Brule, Martonvásári 4, Disponent and Bezostaya 1 were the most frost-resistant and GK Szeged, GK Kincső and Martonvásár 2068 the most frost-sensitive.

The variance analysis of combining ability indicates that in the F_1 generation the variance of both general (GCA) and specific (SCA) combining ability was significant (Table 3). It can be concluded from this that both additive and non-additive gene effects are important for frost resistance. The GCA:SCA ratio (25.4 in the F_1 generation) reveals a prevalence of additive genetic variance compared with dominance and epistasis. The general reciprocal effects were not significant.

Table 1

Variance analysis table for parents and their F_1 hybrids

Source of variance	df	SS	MS	F
Replication	3	17200	5740	
Treatment	143	560000	3920	20.9*
Error	429	80000	187	

* Significant at the 0.1 % level of probability

Table 2

Frost resistance values (survival %) of parents and their F_1 hybrids and the magnitude of Wr–Vr and Wr+Vr

Parents	Bez. 1	Mv 4	Mv 8	Mv 2068	Mv 10	GK Kincső	GK Szeged	Rivoli	Disponent	Brule	B.1201	Cheyenne	Wr–Vr	Wr+Vr
Bez. 1	**87.0**	96.3	97.5	86.0	83.4	72.3	38.8	88.8	92.4	93.5	96.3	93.8	155.0	510.6
Mv 4	89.9	**88.8**	87.5	77.0	75.6	81.0	67.4	81.9	88.7	90.0	83.8	97.4	166.7	386.7
Mv 8	89.5	89.9	**63.4**	52.2	52.5	32.4	20.0	69.5	88.2	92.2	71.6	76.6	122.4	1305.8
Mv 2068	70.0	89.9	74.5	**24.7**	11.5	1.3	5.5	15.9	61.3	85.9	59.4	84.4	–17.1	2008.4
Mv 10	72.5	73.0	58.8	19.3	**28.8**	11.3	1.3	50.0	68.3	74.5	57.5	82.5	52.7	1865.2
GK Kincső	71.5	70.0	40.9	16.7	8.1	**10.0**	1.3	2.5	55.7	70.9	42.5	76.1	29.6	1980.8
GK Szeged	58.8	48.8	7.9	0.0	0.0	3.8	**9.1**	2.6	1.4	58.8	16.3	87.5	–84.9	1718.7
Rivoli	85.4	90.0	69.8	57.0	42.5	8.8	0.0	**25.0**	57.2	25.2	67.1	91.3	–35.0	1771.3
Disponent	87.6	89.9	80.0	57.5	82.2	23.3	10.0	72.9	**84.5**	44.5	91.3	96.1	13.1	1308.2
Brule	91.2	90.0	94.9	88.8	83.8	88.4	79.6	58.7	94.1	**92.5**	82.4	91.3	54.6	510.8
B.1201	86.3	93.4	76.3	73.3	62.2	34.4	36.4	43.8	63.6	81.3	**46.3**	94.9	176.6	1314.9
Cheyenne	98.8	95.0	100.0	90.0	88.5	89.9	79.9	83.7	95.0	93.7	87.2	**94.9**	130.6	178.8
Mean													63.7	1238.35
Variance													7447.2	$4.52 \cdot 10^5$

Freezing temperature: –15°C

$LSD_{5\%} = 18.9$
$LSD_{1\%} = 24.9$
$LSD_{0.1\%} = 31.9$

Table 3
Variance analysis table for combining ability and reciprocal effects based on Griffing's 1st model

Source of variance	df	SS	MS	F
General combining ability (GCA)	11	105287	9572	51.3*
Specific combining ability (SCA)	66	24884	377	2.0*
General reciprocal effect (GRE)	11	1237	112	0.6
Specific reciprocal effect (SRE)	55	8592	156	
Error	429	80000		

*Significant at the 0.1 % level of probability

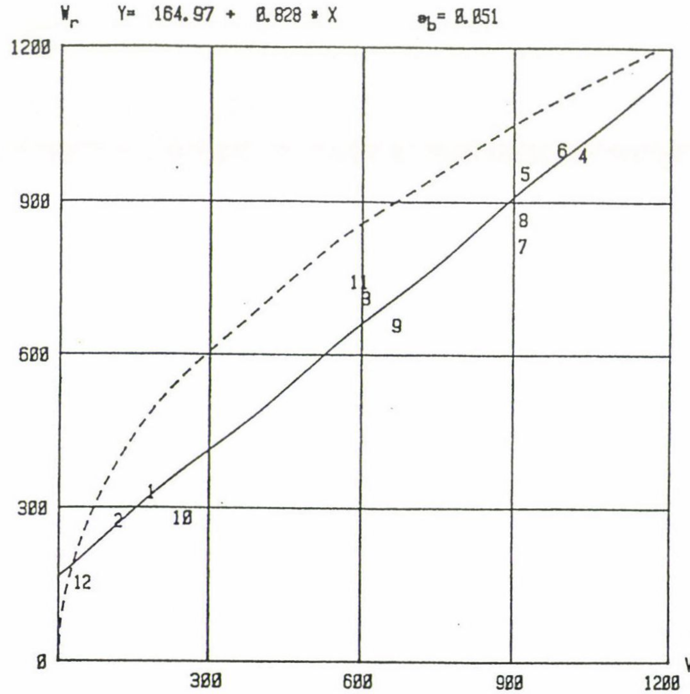
The values of general combining ability effects are illustrated in Table 4. The general combining abilities of the varieties reflected the genotypic values of the parents compared to the diallel mean. The variety Cheyenne had the best general combining ability for frost resistance, followed by Martonvásári 4, Bezostaya 1 and Brule, which had identical values of general combining ability. Among the varieties investigated, GK Szeged and GK Kincső proved to have the poorest general combining ability. Specific combining ability had a negligible effect.

Table 4
Effect of general combining ability on frost resistance

Parents	General combining ability
Bezostaya 1	20.6
Martonvásári 4	21.1
Martonvásári 8	5.4
Martonvásári 2068	−12.2
Martonvásári 10	−12.6
GK Kincső	−25.2
GK Szeged	−36.4
Rivoli	−12.7
Disponent	6.3
Brule	17.2
Bánkúti 1201	1.5
Cheyenne	26.9
S. E.	2.7

The largest quantity of information on diallel crosses can be obtained using the methods of Jinks (1954) and Hayman (1954b), but these models only give reliable results under certain conditions. The existence of these conditions was checked. The variance (Vr) of the individual rows and the covariance (Wr) with the non-recurrent parent were calculated. The correlation of the parental mean and the Wr+Vr values was significant in the F_1 generation. Significant differences were also observed between the Wr–Vr rows, indicating the presence of epistatic effects. The regression coefficient was significantly different from zero for the F_1 population and differed slightly from unity. This again indicates that the non-additive genetic variance consisted of dominant and epistatic effects. Nevertheless, the high $b_{Vr,Wr}$ and low standard deviation values do not justify the rejection of the Jinks-Hayman method.

Figure 1 suggests the partial dominance of frost resistance, since the regression function intersects the Wr axis above the origin and the H_1 value is smaller than D (AB<BD). Cheyenne, Martonvásári 4, Bezostaya 1 and Brule have the lowest Wr and Vr values, i.e. they contain the greatest number of dominant genes, while Martonvásári 2068, Martonvásári 10, GK Kincső, GK Szeged and Rivoli have high Wr and Vr values, indicating that they contain the largest number of recessive genes.



1. Bezostaya 1
2. Martonvásári 4
3. Martonvásári 8
4. Martonvásári 2068
5. Martonvásári 10
6. GK Kincső
7. GK Szeged
8. Rivoli
9. Disponent
10. Brule
11. Bánkúti 1201
12. Cheyenne

Fig. 1. Graphic analysis of the inheritance of frost resistance in the F_1 generation

The calculated values of the genetic components of variance are contained in Table 5. The additive component (D) is greater in the F_1 generation than the dominance component. The degree of dominance, $\sqrt{H_1/D}$ was 0.65 in the F_1, i.e. less than unity, leading to an assumption of partial dominance. The value of $H_2/4H_1$ was close to 0.25, indicating that the ratio of the dominant and recessive alleles in the parents was almost identical. It can be concluded from the negative F values, however, that there is a slight preponderance of recessive alleles in the genetic background of parents influencing frost resistance. The $1/2F/\sqrt{D(H_1-H_2)}$ ratio is less than zero, suggesting that the h/d ratio is not the same at all loci. The values of the narrow sense and wide sense heritability index are relatively high (71.6%, 82.2%). Since the additive gene effect contributes to genetic variance to a greater extent than dominance, and the heritability index is also high, selection in the progeny generation of the diallel cross can be begun at an early stage.

Table 5

Estimation of gene effects in a 12-parental diallel cross (after Hayman, 1954)

Genetic parameters	Estimated values
D	1041.57
H_1	444.89
H_2	443.23
F	–458.77
E	187.00
$\sqrt{H_1/D}$	0.654
$H_2/4H_1$	0.249
$1/2F/\sqrt{D(H_1-H_2)}$	–5.523
Heritability index: Narrow sense	71.64%
Wide sense	82.21%

Discussion

In the present experiments the regression function intersected the Wr axis above the origin and the value of H_1 was lower than D, leading to the conclusion that frost resistance was partially dominant, as established by Eunus et al. (1962) and Rohde and Pulham (1960). Cheyenne, Martonvásári 4, Bezostaya 1 and Brule had the lowest Wr and Vr values, i.e. they contained the greatest number of dominant genes, while Martonvásári 2068, Martonvásári 10, GK Kincső, GK Szeged and Rivoli had high Wr and Vr values, indicating that they contained the largest number of recessive genes. The data reveal that in the case of freezing at –15°C frost resistance was dominant over frost sensitivity. This result contradicts some of the data reported in the literature. In experiments carried out by Puchkov and Zhirov (1978), Sutka (1981) and Beke and Sutka (1983) dominant genes led to poorer frost resistance and recessive genes to greater resistance.

Gullord et al. (1975) suggested that the freezing intensity and the growing conditions might have a substantial modifying effect on the frequency distribution of the segregating generation. Freezing at -6 or −7°C led to a different distribution than testing at lower temperatures (−14 and −18°C). It would seem that different genes determine the response to freezing at different intensities. Although non-segregating generations were tested both by the authors cited above and in the present experiments, one reason for the discrepancy in the present case could be differences in the magnitude of the dominance effect in the varieties included in the diallel cross.

In previous experiments the effect on frost resistance of the 5A chromosomes of varieties with different degrees of frost resistance (Cheyenne, *Triticum spelta*, Hope) when substituted into the same genetic background (Chinese Spring) and on how this was inherited was analysed in the F_1, F_2 and BC progeny generations at various freezing temperatures (Sutka and Veisz, 1988). The 5A chromosomes tested carried different alleles for frost resistance. The additive gene effects were greater than the dominant gene effects for all three parental pairs and at all the freezing temperatures, in agreement with the results obtained in the present diallel analysis and in diallel analyses carried out on varieties by Gullord (1974) and Sutka (1981). The dominance relations clearly depend on what genotypes are tested and on what types of alleles are prevalent in the tested varieties.

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CHANGES IN MAGNESIUM CONTENT OF TREE SPROUTS, DETERMINED MONTHLY BY ATOMIC SPECTROSCOPY

A. CSIKKEL-SZOLNOKI[1] and S. A. KISS[2]

[1]DEPARTMENT OF INORGANIC AND ANALYTICAL CHEMISTRY, ATTILA JÓZSEF UNIVERSITY, SZEGED H–6720, DÓM TÉR 7, HUNGARY
[2]HUNGARIAN MAGNESIUM SOCIETY, SZEGED H–6726, FŐ FASOR 73, HUNGARY

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The deep winter rest of trees can be deduced from external signs, but this involves many problems. A new procedure has been developed for determining the time of deep rest. It was presumed that if the magnesium content in the blood of animals changes during deep sleep in winter, the magnesium content in plants may change likewise.

At monthly intervals over a period of three years, tree sprout samples were taken. These were chopped, dried at 80°C for 48 hours and ashed at 600°C. The ash was dissolved in hydrochloric acid and finally analysed by means of ICP-AES, FES and AAS.

In one group of trees (lilac, hazel-nut and pine), the magnesium content of the sprouts was highest in December. In January the magnesium content decreased significantly (by 17%, 30% and 8%, respectively), which indicated that the deep rest was over. In another group of trees (sour cherry, country maple and plane), the magnesium content of the sprouts (and also that of pine leaves) was highest in January. The deep rest was not over until February, as shown by the decrease in magnesium content (by 32%, 29%, 10% and 17%).

The magnesium content is characteristic in the same types of sprouts and seems to be constant in the same month of the year.

The presumed effect of magnesium during winter sleep (deep rest) is as follows:

The autophosphorylation of protein kinase is increased by increasing amounts of magnesium, but ATP regeneration is blocked. In this way, the formation of cAMP and the activation of protein kinase are decreased, and thus the metabolism may slow down (or stop). Other hormonal effects may also be involved in this process.

These effects may occur both in animals and in plants.

Key words: magnesium, tree sprouts, deep winter rest and sleep, atomic spectroscopy

Introduction

In prolonged periods of cold weather, some groups of animals hibernate, while plants likewise enter a state of deep rest, in which their physiological functions are changed (Hock, 1960).

In autumn and winter, the magnesium concentration in the blood of animals increases significantly (Soumalainen et al., 1938; Aikawa, 1963), by as much as 170% relative to summer levels (Aikawa, 1971). The magnesium content in the blood of the golden hamster increases by 93% following cooling from 25°C to 4°C and the Ca : Mg ratio decreases from 4.77 to 2.81 (Ferren et al., 1971), these changes being needed for the state of rest. Similarly, the pulse rate (Harker et al.,

1987) and the blood pressure of the marmot may decrease by 70% (Zatzman et al., 1987). The blood magnesium level of the squirrel increases in winter sleep (Pengelley et al., 1966), but the pulse rate decreases by 80% when the body temperature falls to 11°C. The corresponding decrease in the pulse rate of the rat is near 100% (Harker et al., 1987), so rats die in much greater numbers than squirrels in response to this low temperature.

There are differences in the magnesium contents in the blood of male and female animals in winter. The blood of the female gopher has a higher magnesium content than that of the male, so the female gopher has better resistance to cold. In summer, there is no difference in the magnesium content (Pengelley et al., 1966).

The defoliation and periodic sleep (dormancy) of trees are internal characteristics that have developed in response to the external climate in accordance with the rhythm of the seasons during evolution. As proof of this internal genetic character, many plants shed their leaves in winter even in a hothouse or in the tropics.

There are states of deep and of forced sleep for plants.

In both state plants do not form shoots, even though they have favourable conditions for growth in deep sleep. Forced sleep follows deep sleep in most cases. Not only different types of plants, but also different parts of the same plant have different deep sleeping times. For example, the male and female flowers and the buds of hazel-nut undergo deep sleep at different times.

Various studies have been made about changes in plants during hibernation, primarily with regard to the contents of organic compounds, e.g. carbohydrates and lipids (Borochov et al., 1989; Dzhnumov et al., 1982; Olien and Lester, 1985; Szalai, 1974), hormones (Volkova et al., 1981) and enzymes (Weidner and Salisbury, 1974). After deep rest, the metabolic processes and activities of stimulating hormones (auxin, gibberellin) increase in trees, and the effect of growth inhibition (dormin) decreases. In deep rest, this takes place in an inverse manner (Gencsi, 1980).

Increasing magnesium content increases plant resistance to cold. This finding agrees with the report (Gromiko, 1968) that a higher mineral salt content (e.g. magnesium) increases the resistance of tomato plants to cold. In deep sleep, apple tree sprouts can tolerate –50°C; in the active state, they can tolerate only –5°C (Larcher, 1981). At low temperature, the uptake of potassium decreases and the uptakes of calcium and magnesium increase (Uilles, 1957). It has been claimed (Wareing and Phillips, 1982) that the resistance of trees to cold increases in deep rest in consequence of the accumulation of organic compounds (carbohydrates and lipids).

The sleeping time of trees can be deduced from external symptoms (swelling of the buds, or sprouting of the branches at room temperature). However, this involves many problems. A new procedure has been developed for the determination of sleeping time. It was presumed that if the magnesium content in the blood of animals changes during deep sleep in winter (Aikawa, 1971), the magnesium concentration of plants might change similarly.

It was postulated that the magnesium concentration of tree sprouts was high during winter, and that it decreased when the winter sleep was over. The correctness of this supposition was confirmed by analytical data on sprouts from different types of trees. Sprout samples were taken every month during a period of three years (Kiss et al., 1994).

Materials and methods

The magnesium contents in sprouts of hazel-nut *(Corylus avellana)*, sour cherry *(Cerasus vulgaris)*, lilac *(Syringa vulgaris)*, country maple *(Acer campestre)* and plane (*Platanus hispanica*), and in the sprouts and leaves of pine (*Pinus sylvestris*), were determined monthly. Both the macroelements (magnesium, calcium, potassium) and microelements (lead, copper, zinc, iron, manganese) were investigated in plane tree sprouts of different ages.

The solutions used for the measurements were made from Merck quality reagents. A matrix modifier was used when necessary (for example, lanthanum reagent for the calcium and magnesium measurements by the AAS method). Rye-grass (CRM 281) was the standard material for the analysis of many elements.

Instrumentation

The elemental analyses were performed by means of atomic spectroscopy. The instruments used were a Jobin Yvon 24 sequential ICP (copper, zinc, iron, manganese), a Jenway PFP7 flame photometer (potassium) and a Carl Zeiss Jena AAS (ETA method for lead, flame method for calcium and magnesium).

Procedures

Digestion method

Sprout samples (branches) collected from different types of trees at monthly intervals between June 1991 and May 1994 were dried at 80°C for 48 hours in a drying box. Average samples were made from five branches of three trees of the same type in every case.

In the initial stages of this study, the sprout samples were then treated by two methods: wet digestion with a mixture of sulphuric acid and hydrogen peroxide (Csikkel-Szolnoki, 1996) and dry treatment by ashing at 600°C, followed by dissolution in hydrochloric acid (Csikkel-Szolnoki et al., 1994). It was found that lower results were obtained for calcium, for example, by the wet method, because of the precipitation of calcium sulphate, and accordingly further analyses were performed only after ashing.

Every measurement was repeated three times.

Physiological experiments

If the leaf and flower buds of woody plants (trees and bushes) are in a state of forced rest after deep sleep, the plants do not form shoots, in consequence of the low external temperature. If the branches are cut off, placed in 30°C water for 10–12 hours and then kept at room temperature, budding and flowering begin only after the state of deep sleep has been completed (Hortobágyi et al., 1963; Kiss, 1992). Accordingly, forcing experiments were carried out on selected plants at different times, in order to establish the end of deep sleep and the beginning of forced rest.

Results

It was established that the deep sleep of sprouts of lilac, hazel-nut and pine occurred in December, as their magnesium contents were maximum at that time. In January, the magnesium concentrations of these sprouts were 8–30% lower, indicating the end of deep sleep (Table 1/a).

Pine leaves, and sour cherry, country maple and plane sprouts were in deep sleep in January, but this was over in February, as indicated by a 10–32% decrease in the magnesium concentration (Table 1/b).

Table 1/a

Changes in magnesium content in 1-year-old sprouts of different trees (mg/kg)

Trees	Years	Months											
		VI	VII	VIII	IX	X	XI	**XII**	I	II	III	IV	V
	1991–92	602	621	642	660	742	770	**880**	695	650	620	601	548
Lilac	1992–93	583	610	620	644	729	754	**815**	600	609	586	595	571
sprouts	1993–94	575	584	591	651	700	731	**790**	750	685	650	538	510
	average	587	605	618	652	724	752	**823**	682	648	619	578	543
	1991–92	562	585	596	597	640	828	**1090**	687	660	625	590	540
Hazel-nut	1992–93	524	549	526	500	553	1000	**1130**	609	644	602	548	525
sprouts	1993–94	553	576	550	611	735	911	**1020**	986	780	690	600	580
	average	546	570	557	569	643	913	**1080**	760	695	639	579	548
	1991–92	625	599	632	640	652	681	**717**	687	593	585	605	581
Pine	1992–93	600	590	610	600	620	668	**789**	647	550	600	610	600
sprouts	1993–94	576	624	591	621	659	700	**810**	790	664	631	581	612
	average	600	604	611	620	644	683	**772**	708	602	605	599	598

Table 1/b

Changes in magnesium content in 1-year-old sprouts or leaves of different trees (mg/kg)

Trees	Years	Months											
		VI	VII	VIII	IX	X	XI	XII	**I**	II	III	IV	V
	1992–93	620	610	640	680	741	820	911	**931**	786	720	650	590
Pine leaves	1993–94	646	620	659	741	760	773	840	**986**	814	792	711	681
	average	633	615	650	711	751	797	876	**959**	800	756	681	636
	1991–92	498	550	699	870	884	917	1640	**1930**	1310	1186	1024	600
Sour cherry	1992–93	526	633	780	928	972	1095	1470	**1800**	1230	1120	957	668
sprouts	1993–94	610	683	801	950	1010	1490	1780	**1850**	1285	1030	1002	700
	average	545	622	760	916	955	1167	1630	**1860**	1275	1112	994	656
Country	1991–92	473	500	538	552	573	600	650	**853**	620	508	520	470
maple	1992–93	480	490	510	500	500	576	590	**816**	570	464	466	445
sprouts	1993–94	535	515	532	535	596	600	610	**874**	631	582	504	593
	average	496	502	527	529	556	592	617	**848**	607	518	497	503
	1991–92	768	760	802	798	850	889	960	**1150**	1000	892	770	758
Plane	1992–93	714	756	722	698	766	789	914	**1100**	981	944	785	748
sprouts	1993–94	692	714	768	800	833	840	986	**896**	950	817	791	701
	average	725	743	764	525	816	839	953	**1082**	977	884	782	736

Table 2
Mineral compositions of plane tree sprouts of different ages (mg/kg*)

Element	Age of sprouts		
	2 years old	1 year old	New sprouts
Mg	880	1050	1100
Ca	6600	6400	5000
K	1250	4000	7800
Pb**	2.4	0.7	0.4
Cu	6.7	8.4	12.9
Zn	11.2	13.5	14.1
Fe	120	81.5	73.1
Mn	6.3	6.9	8.5

* averages
** The trees are situated near a busy street

The concentrations of magnesium and other elements differed greatly in young and older sprouts (Table 2). It is important, therefore, to collect sprouts of the same age for the investigations.

The conclusions drawn from the analytical results were confirmed by the results of physiological experiments. When the samples were collected for the analyses, 10 pieces of the sprouts were forced at the same time (Materials and methods). The forcing was only successful if the magnesium concentration had decreased significantly in the plants. Forcing was successful in 70–80% when the same type of tree sprouts was used, which shows the uncertainty of the physiological method.

Discussion

A new, accurate method has been developed for establishing the deep rest time, by determining the magnesium concentration of tree sprouts (branches).

On the basis of the end of deep rest and the spring germination, the trees and bushes examined fell into two groups: early (lilac and hazel-nut) and late (sour cherry, country maple and plane). This fact was confirmed by determinations of the magnesium contents in different plants.

The maximum magnesium contents showed the time of deep rest in pine sprouts to be in December, whereas that for pine leaves was in January. Thus, different parts of the same plant may have different deep resting times.

A parallel was found between plants and animals, as reflected by the changes in magnesium concentration during winter sleep.

The change in magnesium content did not exactly parallel the change in total cations during deep sleep (Kiss et al., 1992). There is a similar magnesium increase in the blood serum of animals in winter sleep; this increase may be as much as 170% relative to summer samples (Aikawa, 1971).

The magnesium increase in plants is 30–240%, depending on the species.

Plant resistance to cold may be increased by high protein contents (Trunova et al., 1977), while protein synthesis may be accelerated by magnesium (Kiss, 1983).

The presumed mode of action of magnesium during winter sleep (deep rest) involves the fact that protein kinase autophosphorylation increases with increasing magnesium content, which leads to inactivation of the enzyme (Elődi, 1983; Gombkötő and Sajgó, 1985; Kiss, 1983; Müller, 1973). As ATP regeneration is blocked, the formation of cAMP, which would activate the kinase, also decreases. In this way, the metabolism becomes slower (or stops).

Changes in the magnesium contents of sprouts during deep sleep were determined in this work. It should be mentioned that magnesium does not cause direct effects. The change in the magnesium content acts primarily in an indirect way on the activities of the enzymes and hormones during winter sleep (deep rest) and waking (germination).

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GROWTH AND PHOSPHORUS NUTRITION OF MYCORRHIZAL MAIZE PLANTS AT DIFFERENT SOIL VOLUMES AND PHOSPHORUS SUPPLIES

K. POSTA[1] and G. FÜLEKY[2]

[1]DEPARTMENT OF MICROBIOLOGY; [2]DEPARTMENT OF SOIL SCIENCE AND AGRICULTURAL CHEMISTRY; GÖDÖLLŐ UNIVERSITY OF AGRICULTURAL SCIENCES, H-2103 GÖDÖLLŐ, HUNGARY

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The effect of vesicular-arbuscular (VA) endomycorrhizae on plant growth and P-uptake was studied at different rates of phosphorus amendment and different soil volumes under greenhouse conditions. Maize plants (*Zea mays* L. cv. Pioneer) were grown for six weeks in a pot experiment using calcareous sandy soil and three phosphorus rates (0, 50, 250 mg P kg^{-1} soil, marked P0, P1, P2) varying from deficient to supraoptimal for the plant.

Either the soils were inoculated with VA endomycorrhizal fungi (MO+VA), or the plants were raised without VA inoculation (MO-VA). Different volumes of soil were made available to the plants by using pots with different volumes (0.5, 2.0, 5.0 and 10 litres).

The positive effect of mycorrhizal inoculation on the shoot growth declined as phosphorus fertilization was increased. For the various soil volumes, the effect of mycorrhizal inoculation on the shoot mass rose as the soil volume increased.

In all cases, mycorrhizal plants had higher P contents. The positive effect of mycorrhizal inoculation on plant phosphorus uptake was exhibited even when phosphorus was applied. Phosphorus fertilization reduced the degree of root colonization and also the quantity of external hyphae. The results suggest that mycorrhizal inoculation had different effects when using different pot volumes, thus explaining the differences between results obtained in field and pot experiments.

Key words: arbuscular mycorrhiza, root colonization, soil volume, phosphorus nutrition

Introduction

Inoculation with VAM (vesicular arbuscular mycorrhiza) fungi has been shown to affect plant growth, primarily by increasing the uptake of phosphorus (Sanders and Tinker, 1973; Tinker, 1975; Marschner and Dell, 1994)

An increase in the absorption of P by mycorrhizal plants could be brought about by increased exploration of a larger soil volume, modification of the root environment and the efficient utilization of P within the plant (Bolan, 1991).

Many environmental and edaphic factors can influence the development of mycorrhizal colonization in a root system. Two of the most important factors involved in controlling root colonization are the soil and the plant phosphorus content.

Phosphorus fertilizers have varied effects on VAM symbiosis and on the fungi themselves (Abbott and Robson, 1985). The effects appear to be mediated by the plant at low and medium levels of soil P, but directed by the soil at high soil P levels (Thomson et al., 1991). Root colonization may be reduced at high or very low P availabilities (Amijee et al., 1989), whereas spore production is generally depressed by phosphorus availability above the levels at which the host plants benefit from VAM colonization (Menge et al., 1978; Nelson et al., 1981). On the other hand, Miranda et al. (1989) and Miranda and Harris (1994a) observed that the soil phosphorus effect would probably be more evident when the mycorrhiza fungus is first developing in the soil.

The uptake of nutrients by plants is affected by chemical and physical parameters such as the quantity of nutrients per unit of soil and the volume of soil around the roots.

Root competition may influence mycorrhizal activity too. It has been shown that, for plants grown in pots, the volume of soil available to the plant affects its uptake of phosphate (Larsen and Sutton, 1963; Allsopp and Stock, 1992).

Baath and Hayman (1984) and Koide (1991) reported that mycorrhizal infection was the greatest at the lowest plant density. At higher plant densities, fewer differences were observed between mycorrhizal and non-mycorrhizal plants, as the phosphorus depletion zones of their roots overlapped.

There are some reports of VAM inoculation improving yields of field crops such as wheat (Khan, 1975) and soyabean (Kuo and Huang, 1982), but our present knowledge on the role of VA mycorrhizas in plant growth is based on the results of pot experiments.

Weber et al. (1992) used agricultural soils in a pot experiment, and VAM colonization often leads to an increase in plant growth and P-uptake. However, differences have been shown between the results obtained in field and pot experiments.

The ecology of mycorrhizal systems will have to be better understood before VAM fungi can be applied effectively as inoculants. In the present work, varying pot volumes were used to reduce the soil available for root growth and investigate the effects of VAM on growth and the uptake of phosphorus by maize at different soil volumes and P supply levels. Our aim was also to clarify the contradiction between pot and field experiments.

Materials and methods

Cultivation of plants

Pot experiments were carried out in a greenhouse during the summer. Soil samples were collected from the Őrbottyán experimental station (Hungary). The chemical and physical properties of the unsterilized soil were as follows: pH 7.1 in KCl, humus content 0.94%, $CaCO_3$ content 5.5%, Olsen-P 12 mg kg^{-1}. The soil was sieved (2 mm) prior to sterilization to remove stones and coarse plant residues. For sterilization air-dry soil was heated at 120°C for 48 h in an oven, then the soil was left for 6 weeks before using. Different extents of root growth were produced by using pots of the following sizes: 0.5 L, 2.0 L, 5.0 L and 10.0 L. The bulk soil density in the pots was adjusted to 1.2 g cm^{-3}. Three levels of phosphorus (0, 50 and 250 mg P kg^{-1} soil, marked P0, P1 and P2, respectively) were used for the P treatments in the form of $Ca(H_2PO_4)_2 \cdot H_2O$ solution.

Each pot was fertilized with 200 mg N as $Ca(NO_3)_2$, 200 mg K as K_2SO_4 and 50 mg Mg as $MgSO_4$ per pot. Iron, zinc and copper were supplied at a rate of 1 mg pot^{-1} in the form of ferric-ammonium-citrate, $ZnSO_4$ and $CuSO_4$, respectively.

Maize (*Zea mays* L. cv Pioneer) seeds were surface-sterilized using 30% H_2O_2 for 10 min and subsequently washed with sterile distilled water. Four seeds were planted in each pot and were thinned to two plants per pot after emergence. Water was supplied daily at close to field capacity (about 15%) during the growth period on the basis of weight measurements.

The treatments included inoculation with rhizosphere microorganisms together with a VA mycorrhizal fungus [*Glomus mosseae* (Nicol. Gerd.) Gerdemann and Trappe] (MO + VA) or inoculation with rhizosphere microorganisms other than VA mycorrhizal fungi (MO – VA). The inoculum of *G. mosseae* was propagated on maize grown in a greenhouse for 7 weeks. For the production of inoculum containing similar rhizosphere microflora other than mycorrhizal fungi, maize plants were inoculated with a filtered extract (Blue ribbon filter paper, No. 389[3], Schleicher and Schuell, Germany) of the *G. mosseae* inoculum used for multiplication. In both cases, roots with adhering soil were used as inoculum. For the MO + VA treatment, each pot received an equal amount of inoculum (10% of soil volumes, respectively). 10 g mycorrhizal inoculum contained about 400 infective propagules and the corresponding rhizosphere microorganisms. For the MO – VA treatment each pot received an equal amount by weight of inoculum. In all treatments, the inoculum was mixed uniformly with the entire volume of soil in the pots prior to planting.

Each treatment had six replicates.

Plant harvest, plant and soil analysis

The plants were harvested after six weeks of growth. The roots were washed free of soil with tap water and cut into segments approximately 1 cm in length. Root length was measured using the modified line intersect method (Tennant, 1975). An aliquot of washed roots was collected from each pot for the determination of root colonization. The formation of vesicular-arbuscular mycorrhizae was quantified by measuring the arbuscule and vesicule formation by the hyphae of VA mycorrhizae after staining in Trypan blue and lactophenol. The percentage colonization was estimated by the grid-line intersect method (Giovanetti and Mosse, 1980).

The term relative field mycorrhizal dependency was introduced by Gerdemann (1975) and defined quantitatively in the field by Plenchette et al. (1983) as a percentage value:

$$\text{RMFD} = \frac{\text{dry weight of mycorrhizal plants} - \text{dry weight of non-mycorrhizal plants}}{\text{dry weight of mycorrhyzal plants}} \times 100\ \%$$

The fresh weight of external hyphae in the soil was determined using an external mycelium extractor (EME) constructed as suggested by Vilarino et al. (1993).

The EME method promises to be useful for the extraction of fungal biomass, especially for the sandy soil used in this experiment.

The effectiveness of the EME was evaluated using three 50 g samples of soil, divided into 10 g subsamples. 10 g soil was suspended in 75 ml of isotonic NaCl solution, stirred by hand for 20 s, and centrifuged for 5 min at 2000 g. The sediment was sieved (<100 μm) to remove sand particles and root fragments, and washed into a beaker with isotonic NaCl solution. The mycelia were extracted with the EME five times for 3 min each. Mycelia became entangled in the wire framework and were subsequently washed into a beaker with NaCl solution. After the treatment of each 50 g soil, the solution collected was filtered through a 0.45 μm Millipore filter.

The mycelia extracted with the EME from each 50 g sample were dried for 10 min at 40°C on the Millipore filter on which they had been deposited, and the weight was determined as the difference between the total weight and that of the filter alone after the application of NaCl solution and drying under the same conditions. The values were corrected for background values (soil from MO – VA treatments).

The weights of the shoots and roots were determined after drying at 70°C for 72 h. For phosphorus analysis, ground plant samples were ashed at 500°C and digested with 4.7 N nitric acid. Phosphorus was determined colorimetrically according to Gericke and Kurmies (1952).

Analysis of variance was carried out on the data. The least significant differences were calculated (P=0.05) using the T-method (Sokal and Rohlf, 1981).

Results

The effects of the treatments on shoots and roots are given in Table 1. As compared to the non-mycorrhizal treatment, root dry weight was influenced by VAM inoculation only in the biggest pots in non-fertilized soil. Mycorrhizal inoculation did not influence shoot dry weight only in 10 L pots.

The effect of mycorrhizal inoculation on shoot growth could be best observed by plotting mycorrhizal dependence (Fig. 1). The mycorrhizal dependence was generally greater for soils poor in phosphorus, except for the 0.5 L pot size, and was higher in bigger pots. RFMD was most pronounced in non-fertilized soil in 5 L pots, and no differences were found at the P1 and P2 levels in 5 and 10 L pots, respectively.

Mycorrhizal inoculation decreased the total root length in 10 L pots at high soil P, and slightly increased it in non-fertilized soil.

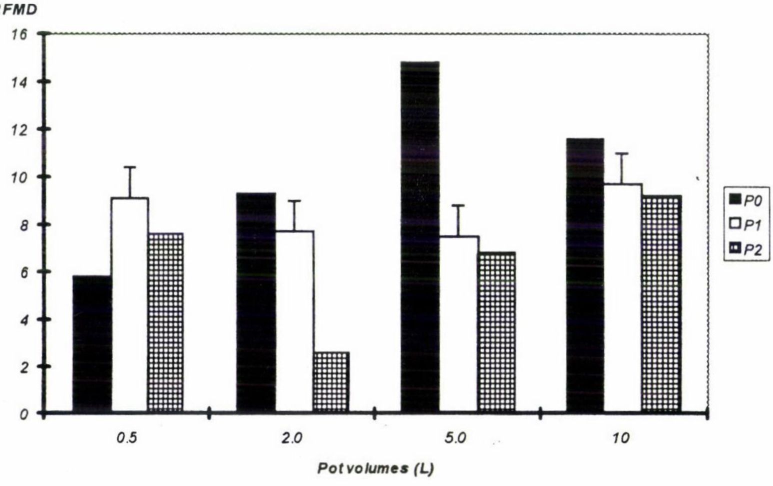

Fig. 1. Effects of pot size and P fertilizer level (P0, P1, P2) on relative field mycorrhizal dependence (RFMD). Bar marker LSD (P = 0.05)

Table 1
Mean parameters of maize plants grown in different pot sizes and at three P fertilizer levels with (+) or without (–) vesicular-arbuscular mycorrhizal (VAM) inoculum

P fertilizer level [mg P kg^{-1} soil]	Pot size [L]	VAM + or –	Dry weight of shoots [g pot^{-1}]	Dry weight of roots [g pot^{-1}]	Root/shoot ratio	Root length [m pot^{-1}]	P concentration in shoots [mg g^{-1}]	P uptake in shoots [mg pot^{-1}]
0	0.5	+	1.7	0.43	0.25	9.6	2.4	4.1
		–	1.6	0.51	0.32	11.1	2.5	4.0
	2.0	+	2.4	0.67	0.27	17.1	3.1	7.6
		–	2.2	0.73	0.33	16.5	2.2	5.0
	5.0	+	2.7	1.80	0.67	35.7	3.6	9.7
		–	2.3	1.86	0.80	21.4	1.8	6.4
	10.0	+	4.3	2.52	0.46	71.5	3.4	19.9
		–	3.8	1.99	0.66	64.7	1.8	6.8
50	0.5	+	2.2	0.35	0.18	10.4	2.9	6.1
		–	2.0	0.35	0.20	13.1	3.5	7.0
	2.0	+	3.9	1.30	0.33	22.2	4.3	15.0
		–	3.6	1.45	0.40	23.6	3.7	9.7
	5.0	+	5.3	3.80	0.72	55.6	3.9	22.4
		–	4.9	2.90	0.59	41.3	2.2	10.8
	10.0	+	9.3	4.90	0.52	115.4	4.3	40.2
		–	8.2	4.65	0.56	145.2	2.1	16.0
250	0.5	+	2.6	0.40	0.15	13.3	3.3	8.5
		–	2.4	0.42	0.17	15.4	3.1	7.5
	2.0	+	3.9	1.79	0.46	25.5	4.7	16.3
		–	3.8	1.89	0.49	31.1	3.0	11.4
	5.0	+	6.9	4.74	0.69	83.2	2.5	18.7
		–	6.5	4.59	0.70	90.8	2.0	13.0
	10.0	+	9.7	5.90	0.61	185.3	3.3	32.1
		–	8.8	5.10	0.69	222.2	1.9	16.0
Analysis of variance:	Soil P effect (A)		**	**	NS	**	NS	**
	Pot sizes effect (B)		**	**	NS	**	NS	**
	VAM effect (C)		NS	NS	*	*	**	**

NS = non-significant; ** = $P < 0.05$; * = $P < 0.1$

The effect of mycorrhizal inoculation on the root and shoot systems is illustrated by the root/shoot ratios (Table 1). The lower root/shoot ratio characteristic of mycorrhizal plants was observed for all soil volumes in non-fertilized soil. At higher phosphorus amendment rates there was little difference in the root/shoot ratios of mycorrhizal and non-mycorrhizal plants. There was no clear effect on the root/shoot ratio due to different pot sizes when phosphorus was added to the soil.

The phosphorus concentration in the shoots was increased 1.2 to 2-fold due to VAM fungi inoculation, except in small pots (Table 1). Soil volume had no clear effect on the phosphorus concentration in mycorrhizal plants. The phosphorus concentration of the plants does not seem to be clearly affected by pot size or by phosphorus amendment to the soil in non-mycorrhizal treatments.

In general, mycorrhizal plants had a higher phosphorus content than non-mycorrhizal plants in all treatments, but no effect was observed in the smallest pots. The greatest difference in the quantity of phosphorus taken up by mycorrhizal and non-mycorrhizal plants was observed for a low-phosphorus soil in 10-litre pots. Like P concentration, phosphorus uptake also increased with increasing P supplies to the soil in 0.5 and 2.0 L pots for mycorrhizal plants.

Different pot size had a marked effect on P uptake by both mycorrhizal and non-mycorrhizal plants. Differences in the uptake of phosphorus due to increasing pot volumes was most pronounced for mycorrhizal treatments.

Root colonization with mycorrhizal fungus varied between 20 and 58% in the mycorrhizal treatment, while no infection was observed in the non-mycorrhizal treatments. The degree of root colonization was significantly reduced by phosphorus fertilization in all pot volumes (Table 2). At identical phosphorus supply levels changes in soil volume did not have a significant effect on mycorrhizal colonization.

Table 2

Effects of pot size and P fertilizer level on root colonization and fresh weight of external vesicular-arbuscular mycelium

P fertilizer level [mg P kg^{-1} soil]	Pot size [L]	Percentage of root infection [%]	Fresh weight of external hyphae [mg 10 g^{-1} soil]
0	0.5	40.3	27.0
	2.0	45.2	11.0
	5.0	45.0	11.0
	10.0	58.5	6.0
50	0.5	25.4	35.0
	2.0	20.3	13.4
	5.0	25.0	5.2
	10.0	30.7	4.5
250	0.5	20.2	4.5
	2.0	15.5	3.5
	5.0	20.1	2.4
	10.0	25.5	2.5
LSD p = 0.05		6.3	1.9

The quantity of hyphae measured in 10 g soil was reduced by an increase in soil volume at the P0 and P1 levels (Table 2). The lower rate of P slightly increased the hyphal mass in 0.5 and 2.0 L pots. The effect of higher phosphorus fertilization in reducing the quantity of external hyphae was observed to the greatest extent in the smallest pots.

Discussion

Maize plants did respond to VAM fungi inoculation in terms of shoot dry weight only in 10 L pots. The lack of shoot growth response to VAM fungi inoculation, particularly at high P supplies, is well documented (Smith et al., 1986).

Similarly, decreased root growth is a common response to the high P nutritional status of the plant when this is combined with mycorrhizal inoculation (Gnekow and Marschner, 1989).

In the present study the root weight of mycorrhizal plants tended to decrease slightly compared to non-mycorrhizal roots, but the root length of mycorrhizal plants was reduced by 17% in 10 L pots at high soil P level. Root morphology was not studied, but Kothary et al. (1990) showed that in maize this root reduction was mainly due to the reduced number of lateral roots. In addition, a reduction in root hair density and length was also observed in mycorrhizal plants by Kothari et al. (1990).

Mycorrhizal fungi is able to improve the uptake of phosphorus by roots by improving the physical exploration of the soil pore space in two ways. Firstly, hyphae adhere to soil particles, which improves contact with the soil solution. Secondly, hyphae can enter smaller soil pores than plant roots and root hairs, so the P depletion zone extended up to 11 cm from the root surface in mycorrhizal plants (Li et al., 1991).

The role of hyphae in increasing the active surface is important when the phosphorus content of the soil is low. There is a three-fold difference in the phosphorus uptake of mycorrhizal and non-mycorrhizal plants in low phosphorus soil and a two-fold difference in soil rich in phosphorus in 10 L pots (Table 1).

The higher acquisition of phosphorus by mycorrhizal plants, in spite of the reduction in root length, suggests the increased efficiency of mycorrhizal roots, which is particularly evident if uptake is expressed per unit root length (data not shown).

However, using a three-compartment system Pearson and Jakobsen (1993) showed that mycorrhizal roots growing together with hyphae did not absorb more P than either non-mycorrhizal roots or hyphae alone. It is possible that the higher hyphal P uptake when no roots are present in the soil (Jakobsen et al., 1994) may be due in part to the fact that there is less microbial competition for P with hyphae but without roots (Jakobsen, 1995).

In the present work the relatively high root densities in the small pots will have resulted in some overlapping of the P depletion zones around the roots. This may have resulted in competition for P between roots and hyphae, so there were no differences in P uptake between mycorrhizal and non-mycorrhizal plants in small pots (Table 1).

High phosphorus applications to the soil depressed mycorrhiza formation and this effect could have been caused by the increased phosphorus concentration in the plant (Amijee et al., 1989; Sanders, 1975) or by the primary effect of soil phosphorus in reducing the external hyphae of VA (Miranda and Harris, 1994a; 1994b). In the present work the external mycelium growth of mycorrhizal fungus increased in smaller pots at low phosphorus supplies (50 mg P kg^{-1}) to the soil; however, the root colonization simultaneously decreased. In this case the reduction in root colonization may have been mediated primarily through the inhibition of intra-radical development of mycorrhizal fungus rather than through the reduction in VAM penetration of the roots (Braunberger et al., 1991).

Abbott et al. (1984) and Miranda and Harris (1994b) also observed a similar effect, but they measured hyphal length and colonized root length. These authors suggest that the effect of phosphorus in decreasing the hyphal length could be due to the increased root growth, which could partly compensate for the decrease in hyphal growth in the soil. In the present work this reasoning alone could not be applied because the total root length was reduced by mycorrhizal inoculation in 10 L pots at high soil P.

Hepper (1983) considers the toxic effect of phosphorus on the fungus to be responsible for the reduction in hyphal mass after phosphorus fertilization, but this view is contradicted by the reduction in hyphal quantity at the same phosphorus level when pot size is increased. So the different hyphae quantities measured at low and moderate soil phosphorus levels cannot be explained solely by the direct effect of the soil phosphorus content on the fungus, but rather by a change in the quantity of root exudate per unit mass due to a change in soil volume. Soluble carbohydrates in the root exudate are considered to be important substrates for mycorrhizal fungi and are therefore important in regulating the formation of mycorrhizal symbiosis.

Large applications of P to the soil did not result in different hyphal masses, indicating the direct effect of soil P on mycorrhizal development, as reported by Thomson et al. (1991).

In addition, a high concentration of soil P may affect soil pH, which has itself been shown to affect the spore germination (Porter, 1982) and hyphal growth (Abbott and Robson, 1985) of mycorrhizal fungus.

Further research will be necessary to determine the effect of a relatively high mass of external hyphae in small pots. The decrease in hyphal density with increasing pot size probably stemmed from their more diffuse distribution in a greater soil volume, or from the inactivity of the hyphae.

The results indicate that the volume of soil available to plants under field conditions is quite different to that available to a plant growing in a pot, which could be one reason for the different results often obtained in greenhouse and field experiments on inoculation with VAM fungi.

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GERMINATION CHARACTERISTICS OF PEA SEEDS ORIGINATING FROM A FIELD TRIAL TREATED WITH DIFFERENT LEVELS OF HARMFUL ELEMENTS

F. NYÁRAI-HORVÁTH[1], T. SZALAI[1], I. KÁDÁR[2] and P. CSATHÓ[2]

[1]INSTITUTE OF AGRONOMY, GÖDÖLLŐ UNIVERSITY OF AGRICULTURAL SCIENCES, H–2101 GÖDÖLLŐ, PÁTER K. U. 1, HUNGARY
[2]RESEARCH INSTITUTE FOR SOIL SCIENCE AND AGRICULTURAL CHEMISTRY OF THE HUNGARIAN ACADEMY OF SCIENCES, H–1022 BUDAPEST, HERMAN O. ÚT 15, HUNGARY

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13 selected micro-elements (Al, As, Ba, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se, Sr, Zn) at 4 or 5 levels (0, 30, 90, 270 and 810 kg/ha element) were applied separately in a long-term field trial, set up on a calcareous chernozem soil in spring 1991. The 13×4=52 treatments were arranged in a split-plot design with 2 replications. Each plot had a total area of 21 m^2. Maize was grown in the first, carrot in the second, potato in the third and pea in the fourth experimental year with the commonly used management techniques.

The phytotoxic effects of the different microelement treatments on yields, on the trace element concentrations of pea grains, and on the germination characteristics of the pea seeds originating from treated plots are discussed in this paper.

Se, As and Cr caused phytotoxic effects on the grain yields, and germination quality also decreased, especially in the Se and Cr treatments.

Key words: heavy metals, germination characteristics, pea, toxicity

Introduction

Heavy metals and other potentially harmful elements are important in several ways: many are used industrially in technically advanced countries, some are physiologically essential for plants and animals, thus having a direct bearing on human health and agricultural productivity, and many are significant as pollutants of ecosystems throughout the world. Heavy metals in soils have received increasing attention in recent years, partly because of the growing scientific and public awareness of environmental issues, and partly because of the development of analytic techniques to measure their concentrations accurately (Alloway, 1990; Csathó, 1994; Pais, 1980; 1991).

Although trace elements, appearing either in cationic or anionic forms, are minor components of the soil's solid phase, they play an important role in soil bioactivity and fertility. The behaviour of trace metals in soils is also related to their origin and source. Trace elements deposited in soils due to human activities may vary greatly in behaviour compared with trace elements of other origin. Metals are a major group of pollutants that are known to have caused environmental problems in agricultural and natural areas (Kabata-Pendias and Adriano, 1995).

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In order to evaluate the movement of some important contaminants in the soil-plant-animal food chain, a long-term field trial involving harmful element loads was set up on a calcareous cherozem soil in spring 1991. The experimental research programme aims at investigating the following topics (Kádár, 1995):

1. The behaviour of these elements in the soil: fixation, availability, leaching, volatilization, transformation.
2. The effect of these elements on soil life: soil biological activity, recording of macro- and microorganisms in the soil, etc.
3. The absorption of these elements by plant roots and their transport within the plants: their accumulation in the shoots, leaves, stems and grains. The effect of the harmful element contents of seeds originating from the treated areas on germination characteristics.
4. The effect of these elements on animals. The plant material derived from the field experiments is fed to animals in feeding experiments conducted by the Department of Animal Nutrition, University of Veterinary Sciences, Budapest, Hungary.

Yields, the trace element concentrations of pea grains, and the germination characteristics of pea seeds originating from the harmful element field trial, as affected by the application of 13 individual trace elements, are discussed in this paper.

Materials and methods

The trial was set up on a calcareous chernozem soil, developed on loess, containing 2.5–3.0% humus and 5% $CaCO_3$ in the ploughed layer. The soil texture is light loam, with 20% clay (< 0.002 mm), 20% silt (0.002–0.02 mm), 40% loess-like powder (0.02–0.05) and 20% sand (0.05–0.25 mm). To ensure a sufficient macronutrient supply throughout the experiment, 100 kg/ha each of N, P_2O_5 and K_2O are applied yearly to each plot. The 13 selected trace elements were applied in spring 1991 at 4 levels. The $13 \times 4 = 52$ treatments are arranged in a split-plot design with 2 replications. Each plot has a total area of 21 m^2. Maize was grown in the first, carrot in the second, potato in the third and pea (variety "Smaragd") in the fourth experimental year with the commonly used management techniques. The treatments and the chemicals used are given in Table 1.

Soil samples were taken each year. Each composite sample consisted of 20 subsamples taken from the ploughed layer of each plot. Plant samples were taken during the vegetation period twice, using 20–40 plants or plant parts per plot randomly. Plant materials (and animal organs) were dried, milled and digested in teflon bombs using cc. $HNO_3 + H_2O_2$ and their total element content was determined, while soil samples were extracted by ammonium-acetate + EDTA (Lakanen and Erviö, 1971), and their available element content was measured, using the ICP technique in all cases. The procedures, materials and methods were described elsewhere (Kádár, 1992).

Seed testing, to determine the germination ability, was carried out according to the Hungarian National Standard MSZ 6354-3 (1992), which conforms with the stipulations given in ISTA Seed Science and Technology Vol. 13, Suppl. 1 and Suppl. 2 from the 22nd Congress, July 1989.

Table 1
Treatments in the field experiment, kg/ha.
Calcareous chernozem, Nagyhörcsök Research Station, 1991 (Kádár, 1992)

Element used	Applied in Spring, 1991				Chemicals used
	0	1	2	3	
Al	0	90	270	810	$AlCl_3$
As	30	90	270	810	$As_2O_3\backslash NaAsO_2$
Ba	0	90	270	810	$BaCl_2 . 2H_2O$
Cd	30	90	270	810	$CdSO_4 . 8/3\ H_2O$
Cr	0	90	270	810	K_2CrO_4
Cu	0	90	270	810	$CuSO_4 . 5H_2O$
Hg	30	90	270	810	$HgCl_2$
Mo	0	90	270	810	$(NH_4)_6Mo_7O_{24} . 4H_2O$
Ni	0	90	270	810	$NiSO_4 . 7H_2O$
Pb	0	90	270	810	$Pb(NO_3)_2$
Se	30	90	270	810	Na_2SeO_3
Sr	0	90	270	810	$SrSO_4$
Zn	0	90	270	810	$ZnSO_4 . 7H_2O$

Results and discussion

The analytic data of the composite soil samples drawn from the ploughed layer of each plot in 1994 are shown in Table 2. The available trace element contents in the soil were determined by NH_4–acetate + EDTA as extractant (Lakanen and Erviö, 1971). The As, Cd, Cr, Hg, Mo and Se concentrations in the control (uncontaminated) treatments remained below the detectable level (<0.1 mg/kg). The original trace element content can be said to be considerable, although within the ranges for uncontaminated soils, for Al: 66; Sr: 40; Ba: 18; Pb: 5; Cu and Ni: 4 mg/kg. The available Zn content was 2 mg/kg by this method. Except for Al, Sr and Ba, all the concentrations increased by one or more magnitudes as an effect of trace element application.

The pea grain yields obtained in the trial were medium to good (2–3 t/ha) in the uncontaminated (control) plots (Table 3). Se, As, Cr and to a slight extent Cd, showed phytotoxic effects on the pea grain yields, in decreasing sequence. In this calcareous chernozem soil the elements forming anions in alkali pH surroundings were the most toxic ones. There were very low and non-detectable yields, respectively, on the plots treated with 270 and 810 kg/ha Se. Yield losses were 80%, 40% and 20%, respectively for the highest dose of As, Cr and Cd (Kádár, 1995).

As an effect of harmful element application, the As concentrations in air-dry pea grains remained undetectable, while Hg only appeared in the grain at the highest level (Table 4). There were only slight increases in the Al and Cu contents. Ba, Cr and Zn values became 2–3-fold higher in the 810 kg/ha

Table 2

Effect of trace element load on the available (ammonium-acetate + EDTA soluble, according to Lakanen and Erviö, 1971) element contents in the ploughed layer, in April, 1994. Calcareous chernozem, Nagyhörcsök Research Station (Kádár, 1995)

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	Available soil trace element concentrations, mg/kg					
Al	66	68	68	67	13	67
As*	4	6	21	80	3	28
Ba	18	27	40	67	6	38
Cd*	14	14	44	164	14	59
Cr	0	1	2	4	1	2
Cu	4	23	65	192	12	71
Hg*	1	2	12	41	6	14
Mo	0	7	8	25	4	10
Ni	4	22	48	86	4	40
Pb	5	29	101	260	19	99
Se*	3	8	33	89	11	33
Sr	40	57	99	314	32	128
Zn	2	21	58	158	9	60

Table 3

Effect of trace element load on the grain yields of pea. Calcareous chernozem, Nagyhörcsök Research Station, 18 July, 1994 (Kádár, 1995)

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	Pea grain yield, t/ha (air-dry yields)					
Al	2.70	2.94	2.72	2.46		2.71
As*	2.40	2.59	2.34	0.43		1.94
Ba	2.51	2.19	2.50	2.81		2.50
Cd*	2.37	2.25	2.33	1.95		2.22
Cr	2.51	1.98	1.93	1.55		1.99
Cu	2.05	2.09	1.95	2.13		2.06
Hg*	2.57	2.76	2.72	2.64	0.79	2.67
Mo	2.89	3.07	2.91	2.71		2.89
Ni	2.85	2.00	2.29	2.37		2.38
Pb	3.27	2.75	2.85	2.53		2.84
Se*	3.41	2.35	0.23	–		1.50
Sr	2.98	2.51	3.39	3.07		2.99
Zn	2.32	2.56	2.41	2.69		2.50

– : there was no detectable grain yield in the treatment.

Table 4

Effect of trace element load on the trace element concentrations of pea grains, mg/kg (in air-dry grains). Calcareous chernozem, Nagyhörcsök Research Station, 18 July, 1994 (Kádár, 1995)

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	Trace element concentrations of pea, mg/kg (in the air-dry grains)					
Al	37.7	29.3	50.8	37.4	73.9	39.2
As*	0.00	0.00	0.00	0.00	–	0.00
Ba	0.71	0.72	2.94	2.26	3.46	1.97
Cd*	0.92	1.08	1.13	1.60	1.14	1.12
Cr	0.25	0.27	0.32	0.44	0.42	0.32
Cu	7.75	9.34	10.14	9.47	0.54	9.65
Hg*	0.00	0.04	0.00	0.48	0.88	0.13
Mo	1.67	102.5	144.5	171.5	67.0	105.0
Ni	2.82	4.39	10.21	12.00	9.35	7.35
Pb	0.00	0.24	0.56	0.25	1.13	0.26
Se*	53.2	123.5	151.0	–	86.0	109.2
Sr	3.27	5.10	7.00	16.75	8.03	9.62
Zn	26.5	46.3	51.4	55.4	15.6	44.9

– : there was no detectable grain yield in the treatment. In the control (untreated) plots As, Cd, Cr, Hg and Pb concentrations were below the detectable level (<0.1 mg/kg)

Table 5

Effect of trace element load on the germination of pea on the 5th day.
Calcareous chernozem, Nagyhörcsök Research Station. Seed testing: Dep. of Agronomy, Gödöllő Agric. Univ., July 1995

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	Germination of pea on the 5th day (%)					
Al	72	70	55	53		62
As*	76	79	67	75		74
Ba	72	78	70	69		72
Cd*	65	62	74	59		65
Cr	80	68	60	58		67
Cu	72	38	62	59		58
Hg*	81	77	52	75	21	71
Mo	60	60	61	59		60
Ni	65	65	70	70		67
Pb	89	83	87	85		86
Se*	30	51	73	–		51
Sr	72	76	67	69		71
Zn	66	69	69	66		67
$LSD_{5\%}$			23			14
Mean	72	69	66	66	6	69

CV% = 15

Table 6

Effect of trace element load on the normal seedling percentage of pea after the 8th day. Calcareous chernozem, Nagyhörcsök Research Station. Seed testing: Dep. of Agronomy, Gödöllő Agric. Univ., July 1995

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	% of normal pea seedlings on the 8th day					
Al	83	80	77	78		79
As*	77	80	69	75		75
Ba	83	81	81	83		82
Cd*	85	87	81	85		84
Cr	82	70	66	67		72
Cu	83	82	81	84		82
Hg*	83	84	61	78	13	76
Mo	81	80	80	80		80
Ni	81	84	79	80		81
Pb	89	85	88	85		87
Se*	30	57	73	–		40
Sr	83	81	70	72		76
Zn	80	81	79	81		80
$LSD_{5\%}$		14				9
Mean	78	79	76	73	4**	76

CV% = 8

Table 7

Effect of trace element load on the abnormal seedling percentage of pea after the 8th day. Calcareous chernozem, Nagyhörcsök Research Station Seed testing: Dep. of Agronomy, Gödöllő Agric. Univ., July 1995

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	% of abnormal pea seedlings after the 8th day					
Al	9	13	16	16		14
As*	13	9	13	13		13
Ba	9	13	9	9		10
Cd*	7	6	10	7		8
Cr	9	15	15	12		13
Cu	9	11	11	11		10
Hg*	9	7	12	10	7	10
Mo	12	14	13	13		13
Ni	11	12	10	7		10
Pb	3	6	6	8		6
Se*	48	18	17	–		21
Sr	9	11	11	12		11
Zn	12	10	11	7		10
$LSD_{5\%}$		7				4
$Mean^{x}$	9	11	12	10	2	11

CV% = 32

Table 8

Effect of trace element load on the dead seedling percentage of pea after the 8th day. Calcareous chernozem, Nagyhörcsök Research Station. Seed testing: Dep. of Agronomy, Gödöllő Agric. Univ., July 1995

Treatment	Elements applied on 22 April, 1991, kg/ha					
	0/30*	90	270	810	$LSD_{5\%}$	Mean
	% of dead pea seedlings after the 8th day					
Al	7	7	7	6		7
As*	10	11	13	12		11
Ba	7	6	7	8		7
Cd*	7	6	7	7		7
Cr	8	15	19	20		16
Cu	7	7	8	5		7
Hg*	8	9	27	13	10	14
Mo	6	6	7	8		7
Ni	7	4	11	12		8
Pb	8	9	6	7		8
Se*	22	23	10	–		20
Sr	7	9	19	16		13
Zn	7	8	10	10		9
$LSD_{5\%}$		10				6
Mean^x	8	8	12	10	3	9

CV% = 51; ^{x}: without selenium

treatments, as compared to the control plots. The increase for Sr and Ni was 4–5-fold, for Mo about 100-fold and for Se about 1000-fold. It can be concluded that Mo and Se may accumulate in the grain almost unhindered, while the Al, As, Hg and Cu contents did not alter, or only slightly, even in the highest treatments (Kádár, 1995).

The germination characteristics of the pea grains harvested on 18 July, 1994 are shown in Tables 5–8.

The germination of peas on the 5th day diminished significantly in the case of seeds originating from plots contaminated by Se or Cr. The same trend was observed in the case of Al and Cu (Table 5). The percentage of normal pea seedlings after the 8th day also decreased significantly due to the effect of Se and Cr toxicity (Table 6). The occurrence of abnormal seedlings after the 8th day was the highest in the case of seeds originating fom the Se, Al, Cr, Mo and As treatments (Table 7). The percentage of dead seedlings after the 8th day increased to the greatest extent in the Se, Cr, Hg, Sr and As treatments (Table 8).

Conclusions

The phytotoxic effect of 13 individual trace metals, applied in spring 1991 in 30–810 kg/ha doses, on the grain yields of pea differed greatly. On the calcareous light loamy soil of the trial, the elements forming anions under alkali conditions were the most harmful ones (selenates, chromates, arsenates, molybdates, etc.).

The seed quality of pea decreased mainly in the plots where grain losses also occurred, as the result of phytotoxicity.

The sharp increase in the trace metal concentration of pea grains due to potentially harmful element application did not diminish the germination quality automatically. In the case of selenium, high grain Se content proved to be harmful for seed quality. A slight increase in seed Cr concentration also proved to be disadvantageous for germination characteristics. A sharp increase in Mo and Zn concentrations, on the other hand, did not affect seed quality adversely.

In the first year of this long-term field trial with harmful element loads, when maize was produced, the seed quality diminished to the greatest extent in the Cr, Mo, Se and Ba treatments: the percentage of normal seedlings decreased from 85 to 40–60% due to the phytotoxic effect of these elements (Kádár, 1995).

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SOIL TILLAGE INFLUENCED BY THE PHYSICAL STATE OF THE SOIL

M. JOLÁNKAI[1], M. BIRKÁS[2] and T. SZALAI[2]

[1]HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST, HUNGARY
[2]UNIVERSITY OF AGRICULTURAL SCIENCES, GÖDÖLLŐ, HUNGARY

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A survey of the physical state of the soil was conducted in Hungary in two different series of experiments between 1986–89 and 1991–94. The agronomic impacts, the means of soil tillage and the effect of three major crops were evaluated. An examination of the cultural state of the soil, its compaction, dust formation and the energy consumption of cultivation were the main objectives. The results suggest that soil compaction in the three experimental locations was more severe in recent years compared to the late eighties. Interactions between soil tillage implements and soil moisture content were found to have the strongest influence on soil parameters. According to the depth of the compacted layers there were unfavourable changes in fuel consumption.

Key words: soil, environment, soil tillage, compaction, cloddiness

Introduction

All over the world there are two major targets for today's agriculture: one is to produce more food, whilst the other is to do so in a more natural way. The latter would mean a reduction in agronomic impacts in most cases (Marsh, 1992; Jolánkai, 1993). Technical activities are seen by the public to be a primary cause of the environmental pollution arising from agriculture. However, without the use of advanced cultivation techniques (Soane and van Ouwerkerk, 1994; Watts and Dexter, 1994), machinery and agrochemicals, we could never have reached the high crop yield levels which contribute to the nutrition of today's world population (Fowden, 1993). Soil tillage is a basic technological process which may determine the result of plant production. On the other hand, it may also induce severe agro-ecological problems (Birkás, 1995; Birkás et al., 1995). Soil tillage and plant nutrition are the two most important crop production factors (Nagy, 1993). Agricultural production is related to the environment. Every technological effort is related to a certain agroecosystem (Szunics et al., 1993). Each field where agricultural production is introduced should be considered as an ecological unit (Király, 1985). This unit is exposed to two major inputs: material and technical ones. During the crop year and as a consequence of harvest, the field produces a certain material export as well. Due to these inputs and outputs there are several positive and negative changes in the state of this ecological unit.

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The consequences of excess agronomic impacts may be: (1) environmental pollution; (2) phytotoxicity; (3) alterations in the coenosis; (4) water and wind erosion; (5) the induction of resistant mutants; (6) yield losses.

On the other hand, insufficient agronomic applications may also have severe consequences: (1) soil abuse; (2) the danger of pests and diseases; (3) alterations in the coenosis; (4) mycotoxins; (5) yield losses.

The aim of our study was to evaluate agronomic impacts, the means of soil tillage and the effect of three major crops in relation to the physical state of the soil. The cultural state of the soil, its compactness, dust formation and the energy consumption of cultivation were the main objects of our study.

Materials and methods

At the Department of Soil Cultivation of the University of Agricultural Sciences, Gödöllő, data on a farm field soil survey over two periods of three years (1986–1989 and 1991–1994) were processed to evaluate the effects of the physical state of the soil on the effectiveness and quality of soil cultivation. Fields with three main crops (maize, winter wheat and sugar beet) were studied. Figure 1 shows the locations in Hungary (Hatvan-Nagygombos, Jászapáti and Szolnok) where the study was conducted. Monitored test tillage was examined in 1994 at one location only (Hatvan-Nagygombos). In each of the experimental years an identical number of farm fields was sampled at a depth of 0–60 cm. The area tested varied from 1220 to 1320 ha according to the survey period and to the given stage of crop rotation.

Randomized experimental sites were investigated with the following methods: (1) exposure of monolites; (2) Nekrasov type sample tubes (Zhukovsky, 1954) to determine bulk density; (3) Cone resistance measured using an Irvine penetrometer (Jóri and Soós, 1990).

All the experimental fields belong to one of three types of soil: (1) chernozem brown forest soil; (2) chernozem; (3) meadow chernozem.

These soil types could be classified physically within the loam range. The average clay content showed only minor differences according to the crops: (1) sugar beet (47–48%, w/w); (2) maize (49–51%, w/w); (3) winter wheat (50–51%, w/w).

The soil tillage implements used in the study were the following: (1) plough (Kühne-Case-IH-10-720); (2) disk (Kühne-Case-IH-10-770); (3) loosener (Rába-Case-IH-10-14); (4) heavy-duty cultivator (Rába-Case-IH-10-6500 Conser Till).

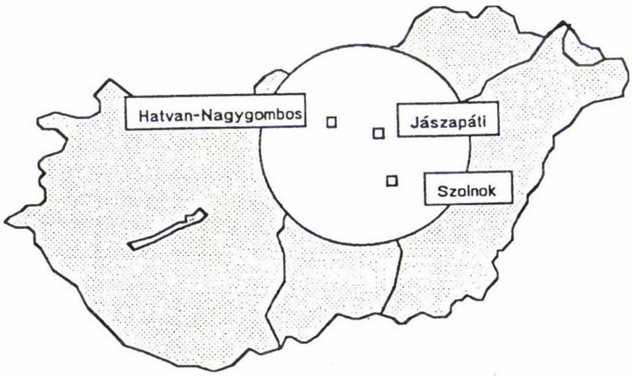

Fig. 1. Experimental locations of the soil survey concerning cultivation practices conducted in the years 1986–1989 and 1991–1994

Results and discussion

Soil tillage is an essential element in production technologies. Distinctions have to be made between the types and depths of cultivation. Soil tillage should be performed within the optimum range of soil conditions, so that undesirable impacts on the soil and the environment do not occur, and crop yields are not risked.

Table 1 presents data concerning the location of compacted soil layers and the frequency of their occurrence. According to the sampling result, ten types of layer formations could be distinguished: (1) non-compacted; (2) 8–12 cm depth single layer; (3) 0–4 and 18–22 cm depth double layers; (4) 0–4 and >40 cm depth double layers; (5) 18–22 cm depth single layer; (6) 18–22 cm and >40 cm depth double layers; (7) 28–32 cm depth single layer, (8) 18–22 and 28–32 cm depth double layers; (9) 28–32 and >40 cm depth double layers; (10) >40 cm depth, deep compaction. In each crop - sugar beet, maize and winter wheat – frequency figures show differences between the test periods. In most cases there was an improvement in soil compaction. The shallower the layer, the more the compaction improvement. The compaction of layers of >40 cm depth did not change much over the years.

Table 1
Location of compacted soil layers and frequency of their occurrence (%) at Hatvan-Nagygombos, Jászapáti and Szolnok, 1986–1994

Depth of compacted layer[a] (cm)	Crops					
	Sugar beet		Maize		Winter wheat	
	1986–1989	1991–1994	1986–1989	1991–1994	1986–1989	1991–1994
Non-compacted	19.5	7.9	18.9	8.1	19.9	3.3
8–12 only	0.6	1.6	0.3	1.4	1.1	1.2
0–4 and 18–22	1.0	2.2	1.3	1.6	1.7	2.3
0–4 and >40	7.1	1.6	6.4	2.5	0.5	1.1
18–22 only	5.3	8.8	6.0	16.7	13.5	28.2
18–22 and >40	4.7	12.3	5.5	7.0	20.0	22.0
28–32 only	10.0	19.0	14.1	16.2	3.2	3.9
18–22 and 28–32	2.8	2.6	4.0	3.7	9.0	6.4
28–32 and >40	20.6	17.0	2.5	14.3	3.1	2.5
>40 only	28.4	27.0	31.0	28.5	28.0	29.1
Area tested (ha)	1320	1230	1300	1320	1220	1230
Clay cont. (% w/w)	48	47	49	51	51	50

[a]Compacted soil means: <40% (v/v) total porosity, or >1.55 g cm^{-3} dry bulk density, or >3.0 MPa cone resistance.

Table 2
Dust formation in relation to the cultural state of the soil at Hatvan-Nagygombos, 1994[a]

Soil	Clod fraction (%, w/w)	
	<5 mm	>5 mm
Regularly cultivated soil, good farming practices	12	88
Occasionally cultivated or abandoned soil	26	74

[a]soil clay content: 49.2% (w/w); soil moisture at 0–2 cm depth: 6.2% (w/w), at 2–32 cm depth: 18.7% (w/w)

One of the most peculiar types of soil extinction or "soil death" is dust formation. At the Hatvan-Nagygombos experimental location, dust formation due to soil cultivation was evaluated in fields in a good or bad cultural state. Table 2 shows the main data of this experiment. The amount of soil particles <5 mm was more than twice as high in the case of bad farming practices, which involved non-regular cultivation or abandoned conditions.

Table 3 presents the data of the 1994 trial conducted at Hatvan-Nagygombos to evaluate trends in cloddiness. Eleven levels of soil moisture were examined (each 2% interval represents the whole ±1% range) during the use of different soil tillage implements (plough, disk, heavy-duty cultivator and loosener). Figure 2 shows the main trends in this study. Plough and loosener induced more cloddiness compared to disk or harrow. However, in the moisture range between 18–24% (w/w), the use of a loosener is safer than that of a plough.

Table 3
Effects of soil tillage implements on the proportion of clods >50mm (%, w/w of dry soil) in relation to soil moisture, Hatvan-Nagygombos, 1994[a]

Soil water content (%, w/w)	Plough		Disk	Cultivator	Loosener
	20–22 cm	28–32 cm	18–21 cm	20–22 cm	40–45 cm
10	45	48	31	25	51
12	42	46	23	22	46
14	39	39	20	19	42
16	34	33	17	15	30
18	30	29	16	14	25
20	28	27	16	15	22
22	26	25	18	17	21
24	27	25	20	19	24
26	32	31	23	22	27
28	37	38	26	24	31
30	46	48	28	27	37

[a]Average clay content: 51% (w/w); dry bulk density before tillage: 1.48 g cm^{-3}

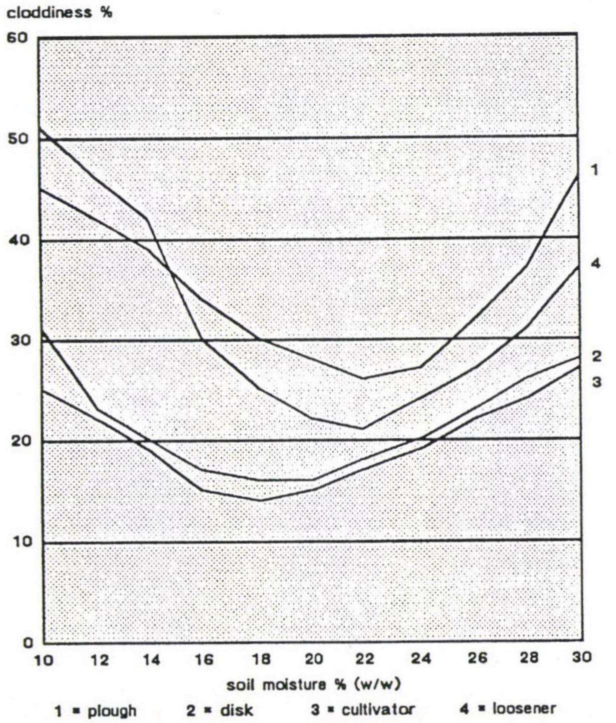

Fig. 2. Trends in soil cloddiness as an effect of cultivation implements, Hatvan-Nagygombos, 1994

Table 4 shows the main data of the fuel consumption experiment. Three implements were tested in fields with different compaction characteristics. In the case of deep layer compaction looseners had a lower fuel consumption compared to ploughing.

Nowadays in most countries of the world many efforts are underway to make agriculture more sustainable, or to run it at a lower input level. However, these attempts face several problems. First of all, compromises have to be made, because none of these systems is quite good enough to solve certain production problems. On the other hand, the natural armoury of agriculture is also restricted. It is nearly impossible to set up healthy, farm-scale crop rotations and to do appropriate soil tillage without technically advanced energy-consuming machinery, or to apply really efficient application treatments with natural substances.

Finally, the results of this study suggest that calibrated methods are needed. Soil tillage has to be done in accordance with the type and the physical and cultural state of the particular farm field. Appropriate soil tillage will not do any harm to the soil or to the whole agroecosystem represented by the given farm field. Since its products are needed for mankind, agriculture is essential. However, we possess all the knowledge and techniques needed for environmentally sound production. Of course, as in all cases of our life, we have to compromise between ecology and economy. Without doubt, there is an optimum balance between the two.

Table 4
Effects of compacted soil layers on fuel consumption ($l\ ha^{-1}$), Hatvan-Nagygombos, 1994

Depth of compacted layer (cm)[a]	Plough 28–32 cm	Loosener 40–45 cm	Cultivator 20–22 cm
8–12	25.7	22.7	15.7
18–22	27.2	27.3	18.8
28–32	26.2	25.4	–
>40	–	28.6	–

[a]bulk density: >1.55 g cm^{-3}, soil clay content: 49.1% (w/w), soil moisture (0–45 cm): 18.0% (w/w).

Conclusions

1. The compacted layers provide information about the depth and method of cultivation, the expected risk for crop production, the expected energy demand and the quality of the result of the next cultivation.
2. The physical condition of the soil at a given time influences the quality of the result of the next cultivation. Compacted soil conditions increase the cloddiness of the result of traditional primary cultivation (ploughing), and thus the need for extra passes in seedbed preparation.
3. The physical state of the soil influences the energy consumption of cultivation. On compacted soils 5–25% more fuel is needed for any primary cultivation procedure. On soils in good physical condition a reduction in the energy demand of cultivation and an improvement in the quality of the result can be expected.
4. The objective of economic and soil-protecting cultivation is the preservation of the favourable physical state of the soil.
5. Rational tillage aims at economic crop production and at soil protection without increasing the risks of farming even in the long term.
6. The main requirement facing soil cultivation systems in rational crop production is that the number and order of cultivation processes should be adjusted to the initial soil conditions. Cultivation errors which destroy the soil and increase production risks should be avoided.

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EFFECT OF N FERTILIZATION ON THE N-CONTAINING GASES OF THE SOIL

K. DEBRECZENI[1], K. FISCHL[1], G. HELTAI[2] and Á. BÁLINT[2]

[1]INSTITUTE OF AGROCHEMISTRY AND SOIL SCIENCE,
PANNON UNIVERSITY OF AGRICULTURAL SCIENCES, KESZTHELY, HUNGARY
[2]DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY,
GÖDÖLLŐ UNIVERSITY OF AGRICULTURAL SCIENCES, GÖDÖLLŐ, HUNGARY

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Recently more and more attention has been given to the need to reduce losses of fertilizer nutrients. This is important both from the economic and the environmental points of view. In the soil, N fertilizer is exposed to movement and transformation, owing to its solubility and its participation in microbiological processes. According to the results of experiments only about 50% of the N fertilizer applied is utilized by the plants. The rest is leached, immobilized or lost through denitrification, depending on the soil characteristics (pH, moisture, biological activity, etc.). A model experiment was set up in pots containing 40 kg of soil taken from the cultivated layer of a clay-illuviated brown forest soil, with maize as test crop or without a crop, using 150 mg/kg NO_3–N and NH_4–N fertilizers (with basic PK) and soil moisture levels of 85% and 65%. Gas-collecting traps were incorporated into the soil at a depth of 20 cm. After starting the experiment, soil air samples were taken from the traps through silicon pipes on the 6th, 20th, 34th, 48th and 68th days. The N gases (NO, NO_2, N_2O, N_2) were analysed by gas chromatography.

Key words: denitrification, N fertilization, gaseous N losses

Introduction

Gaseous N losses from the soil are equally important whether they originate from the continuous ammonification of the organic matter in the soil or from the N fertilizer applied. In this process the N balance tends to get out of equilibrium. According to literary sources field crops utilize 40–60% of the N fertilizer supplied (Colbourn and Dowdell, 1984; Debreczeniné, 1989; Debreczeniné and Szlovák, 1990; Hauck, 1981; Korenkov, 1976; Smirnow, 1979; Kudeyarov, 1989). Depending on the conditions of nitrate leaching, a considerable amount of nitrate nitrogen, occasionally as much as 15–30%, may be lost from the ploughed layer (Smirnow, 1977). In addition 10–25% of the fertilizer may be incorporated in various organic nitrogen compounds of the soil, in the crystal lattice of the clay mineral (NH_4^+), and the extent of gaseous N loss may be 7% for grasses (Colbourn and Dowdell, 1984) and as much as 20% in ploughed fields. According to the results of recent investigations a reduction in the nitrites and nitrates in the soil may also take place under the influence of organic matter in the soil, containing phenol and quinine radicals, and of heavy metals occurring in solution (Smirnow, 1977; Isermann and Henjes, 1989). The rate of transformation of the mineral forms of nitrogen depends on the C:N ratio. In the grass experiments carried out by Simarmata et al. (1991) the quantity of N_2O–N formed per hectare on the second day of the denitrification process was 4.2 kg in a soil fertilized with 300 kg N/ha, 2.9 kg in straw-fertilized soil and 1.9 kg in compost-treated soil. This quantity began to fall in the 4th week until the 40th week and then remained largely at the same level up to the 540th day of the experiment (0.03–0.10 kg N_2O–N/ha/day).

Van Cleemput et al. (1990) studied the effect of maize, barley, potato and sugar-beet leaves and roots and that of humic acid on the denitrification of 100 mg NO_3–N/kg soil (pH=6.8; C=1.26%) in incubation experiments. During the decomposition of pulverized plant leaves the transformation of nitrate was considerably faster than when the roots of the same plants were used. Humic acid had no effect on the nitrate reduction. Studies on changes in the nitrogen gas composition and concentration in the soil air in order to judge the complicated transformation and proportion of loss for various N sources (fertilizer, mineral nitrogen compounds of soil) should be given priority, partly because of their environmental protection implications. Such studies will help in coordinating the nitrogen balance of the soil with the nitrogen demand of plants.

Materials and methods

In model experiments (40 kg dry soil per pot) studies were made on the effect of various forms of N fertilizer (KNO_3, NH_4Cl, 150 mg N/kg soil) on the N balance in the soil-plant system when added to the ploughed layer of lessivated brown forest soil (pH_{KCl}=7.7, C=1.1%, nitrate nitrogen 17.5 mg/kg, NH_4–N 12 mg/kg) with basic PK fertilizer. The experiments were carried out at two soil moisture levels (water holding capacity = 65 and 80%) with and without maize (Volga, Pioneer 3732) as test plant, under greenhouse conditions with 4 replicates, in 1991 and 1992. The experiments were continued for 10 weeks in both years. In 1992 the after-effect of the previous year's experiment was also examined. Gas traps of 1.8 l capacity with silicon pipe outlets were placed at a depth of 20 cm in the soil. In the experiments the parameters of the experimental soil, its mineral nitrogen content and nitrification activity, the weight of plants, their total and nitrate nitrogen content, and the nitrogen gas composition of the soil air were examined (on the 6th, 20th, 34th, 48th and 68th days). The composition of the nitrogen-containing gases (NO, NO_2, N_2O) was determined with a Carlo Erba type apparatus with NP specific detector in nitrogen carrier gas (1.6 bar from a 5 cm^3 sample) as compared to the appropriate calibrating gases, in 3 replicates. The molecular nitrogen was determined with a Carlo Erba model C-type gas chromatograph, thermal conductivity detector in argon carrier gas (0.5 bar). This paper contains the results achieved in 1992.

Results and discussion

The composition of the soil air samples is shown in Table 1 averaged over the nutrient supply and moisture level treatments and expressed in mg N/gas trap. The results of the model experiments are, naturally, to be understood without cover, so the extent of gas diffusion may have been similar to or larger than when under conditions of cultivation. The distribution of the N-containing gases accumulated in the traps shows a considerable variation. With an advance in the vegetation period almost all forms of these gases increased in quantity to a greater or lesser extent as a result of a

Table 1

Quantities and proportions of nitrogen-containing gases per culture pot (N mg/1.8 litre)

Treatment		WHC*	6th day				20th day				34th day				48th day				68th day			
			NO	NO_2	N_2O	N_2	NO	NO_2	N_2O	N_2	NO	NO_2	N_2O	N_2	NO	NO_2	N_2O	N_2	NO	NO_2	N_2O	N_2
with plants	0	70-90%	1	25	5	65	1	28	7	85	2	31	10	91	2	36	10	94	2	37	12	87
		60-70%	0	25	4	61	3	27	7	81	3	31	2	35	2	35	10	89	2	36	10	86
	KNO_3	70-90%	3	32	16	134	7	97	29	389	23	127	44	492	20	140	56	542	28	149	66	605
		60-70%	3	31	13	121	7	95	35	364	18	121	37	482	13	136	51	526	26	143	67	595
	NH_4Cl	70-90%	2	24	13	130	6	93	57	378	17	122	39	456	18	110	42	435	19	107	44	395
		60-70%	1	25	13	118	6	92	32	363	16	117	31	425	12	109	40	431	18	104	43	392
without plants	KNO_3	70-90%	3	15	12	84	13	127	34	309	29	180	55	542	34	186	80	788	37	188	87	883
		60-70%	2	15	10	79	13	123	33	302	31	163	51	556	21	186	72	755	32	185	83	825
	NH_4Cl	70-90%	2	15	10	79	12	125	32	300	24	170	53	576	34	181	78	743	27	183	86	840
		60-70%	1	13	9	66	10	120	28	292	27	165	52	520	17	179	76	700	27	158	84	810
$LSD_{5\%}$			2	14	7	48	5	39	16	92	7	48	17	75	12	19	8	123	12	41	12	80

*WHC: water holding capacity

better water supply. However, according to statistical calculations, the effect of moisture levels is not significant. In the control treatments increases in the amounts of different gas forms were hardly detectable. The amount of molecular N_2 gas was larger in each case, since it is the final product.

The nitrate fertilizer treatment resulted in most cases in the largest volume of N_2 gas in the gas composition, i. e., this is the gas occurring in the largest proportion. More than 30% over N_2 gas could be found in the soil under the plants by the end of the vegetation period, compared to the NH_4Cl treatment. The accumulation of NO_2 gas was the second greatest and this changed similarly during the vegetation period.

In the unplanted pots the production of nitrogen-containing gases became more intensive after sampling on the 20th day, with the consequence that differences due to N fertilizer form disappeared by the end of the experiment. In these pots a nearly 10-fold difference in molecular nitrogen formation could be detected at the end of the experiment compared to pots with plants.

The results show that the NO_2 gas represents the main source of N_2 formation, since it was found at each date of sampling to have a higher rate of concentration in the soil air than the other NOx gases. Under anaerobic conditions the same is characteristic of N_2O.

The total amount of the different N-containing gas forms accumulated in the gas trap in the root zones of planted and unplanted treatments, respectively, can be seen in Figures 1 and 2 in mg N/pot for each N source (soil N, KNO_3, NH_4Cl) and moisture level at each date of sampling.

As seen from the figures the treatments resulted in considerable differences in the amount of gas production. A particularly large volume of N-containing gas formation was found in the soil of unplanted fertilizer treatments. Under the influence of KNO_3 the gas production due to denitrification in the root-zones of the plants was consistently higher than in the NH_4Cl treatment. In the unplanted experiment the difference in gas production between the various N fertilizer treatments disappeared owing to the intensive microbiological processes.

A further way of processing the data of gas composition and quantity obtained during the experiment was to subtract the amount of each gas sample from the value of the subsequent gas sample. From these data of gas accumulation between two dates of sampling conclusions can be drawn on the dynamics of N gas formation. Further, the values of nitrogen gas quantities appearing in the control soil were subtracted from those of the N fertilization treatments, thus giving the more or less corrected amounts of N-containing gas losses caused by the treatments. The data called corrected gas values are shown in Figures 3 and 4. According to the evidence of the figures, N-containing gas formation was most intensive at the 20th day of the experiment. At the 6th and 34th day of sampling considerable amounts of N containing gas were also recorded, while subsequently the gas production due to denitrification decreased and became more or less constant. In response to the KNO_3 treatment relatively high gaseous loss occurred in the planted pots compared with the plant-free ones.

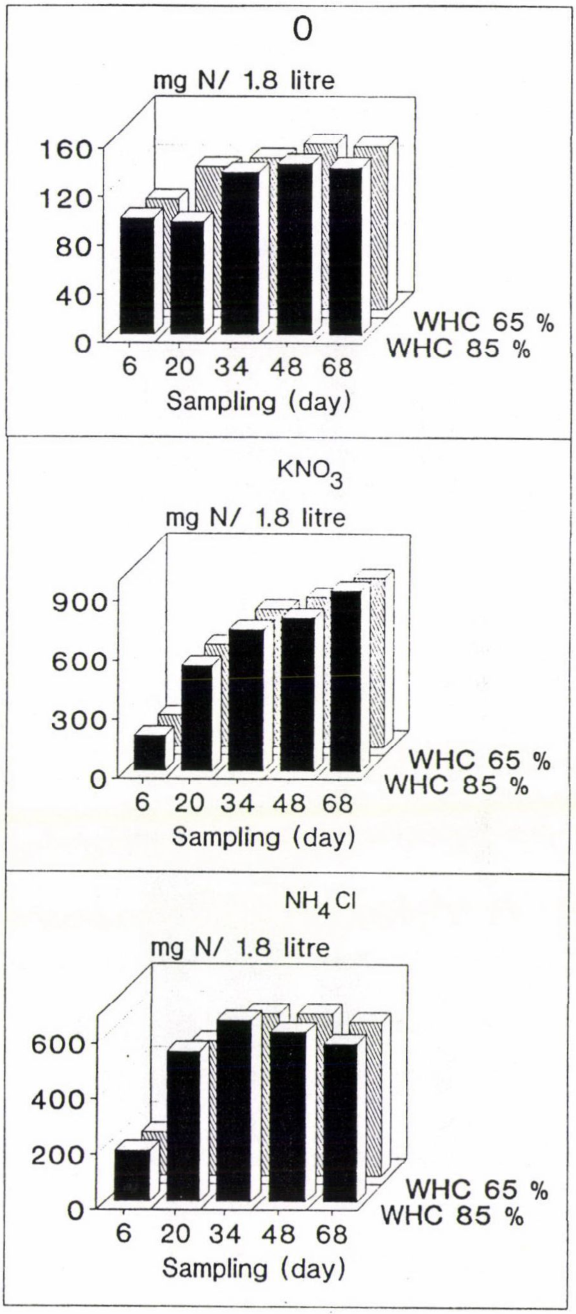

Fig. 1. Gaseous N loss (mg N/1.8 litre) in the root zone as a function of treatment

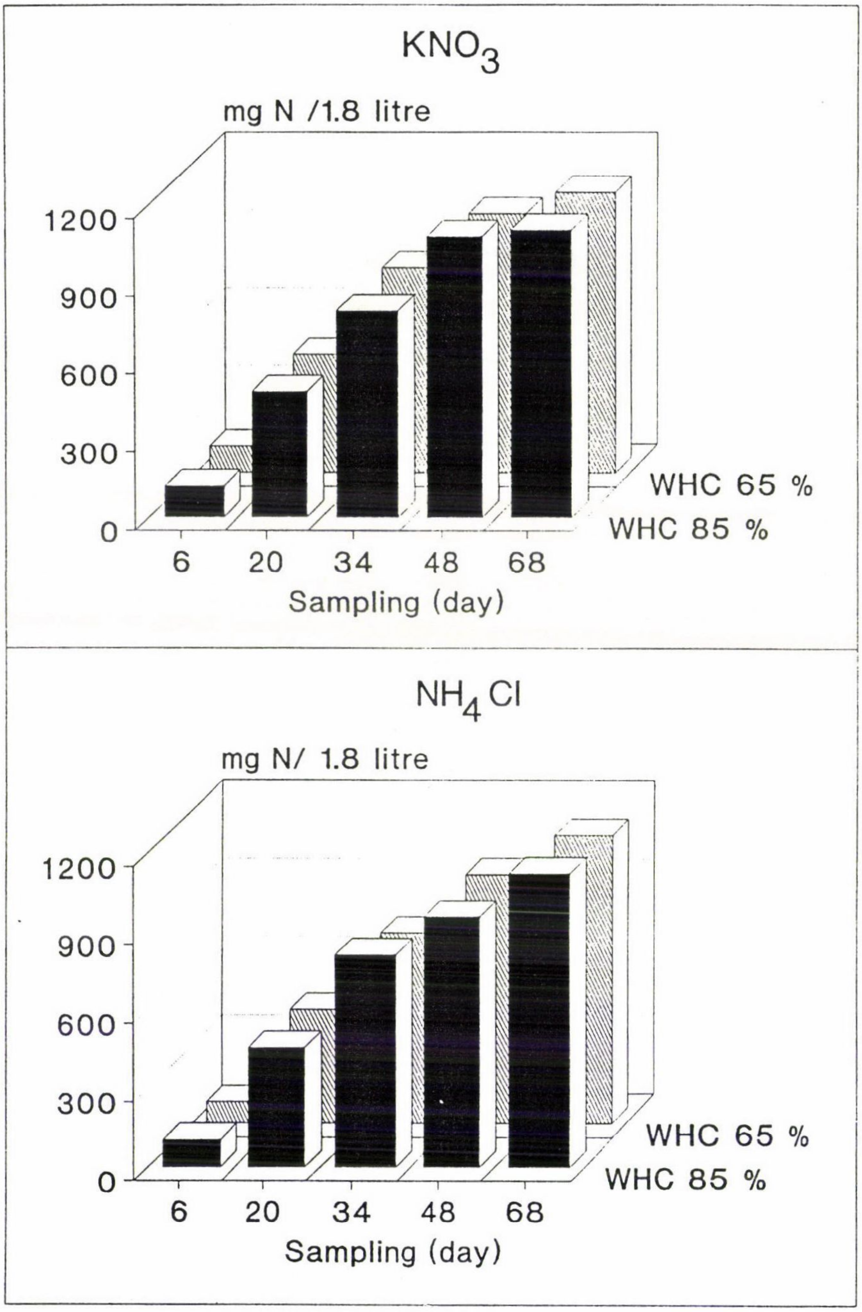

Fig. 2. Gaseous N loss without plants (mg N/1.8 litre) as a function of treatment

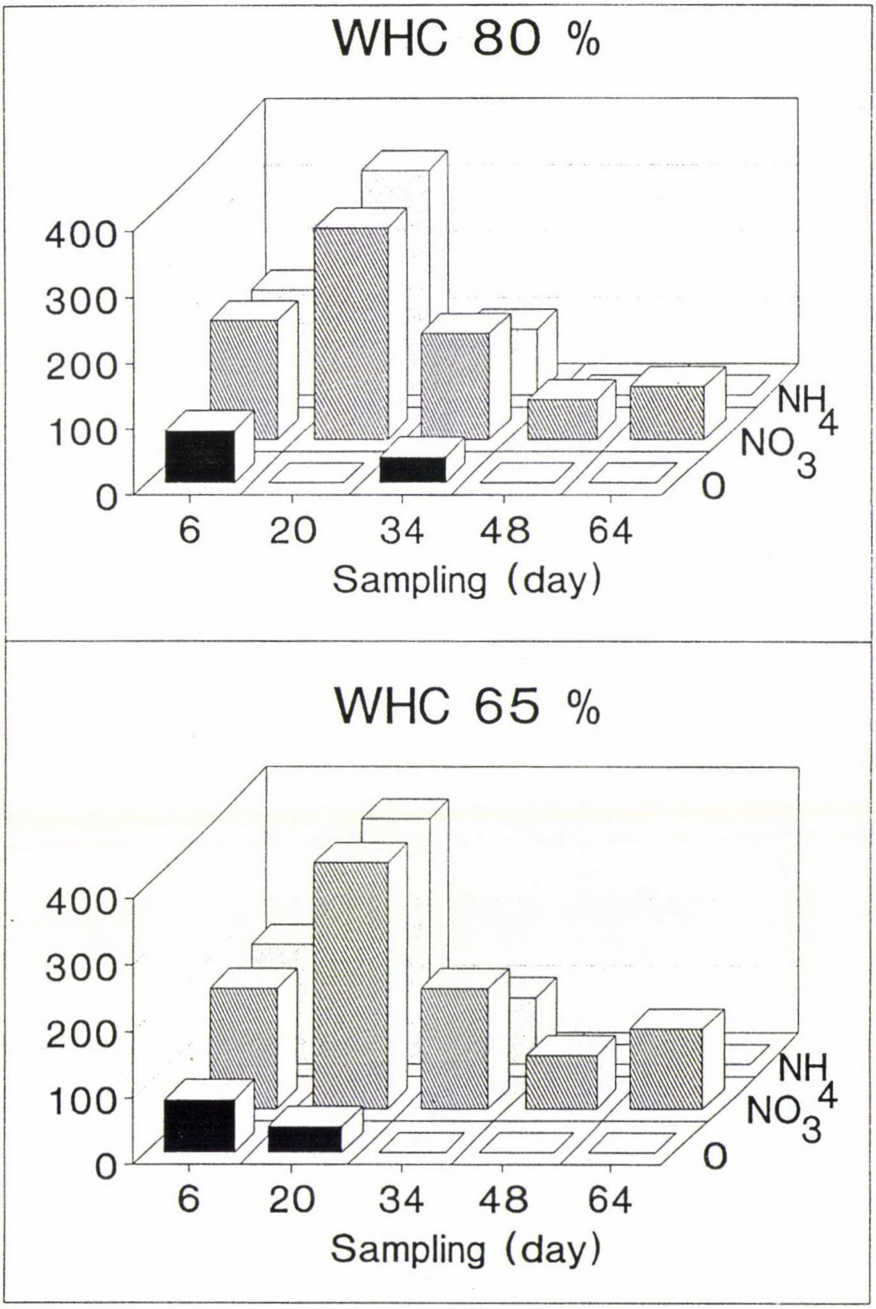

Fig. 3. Corrected values (mg N/1.8 litre) of N-containing gases in the root zone as measured in gas traps in the 1992 experiment

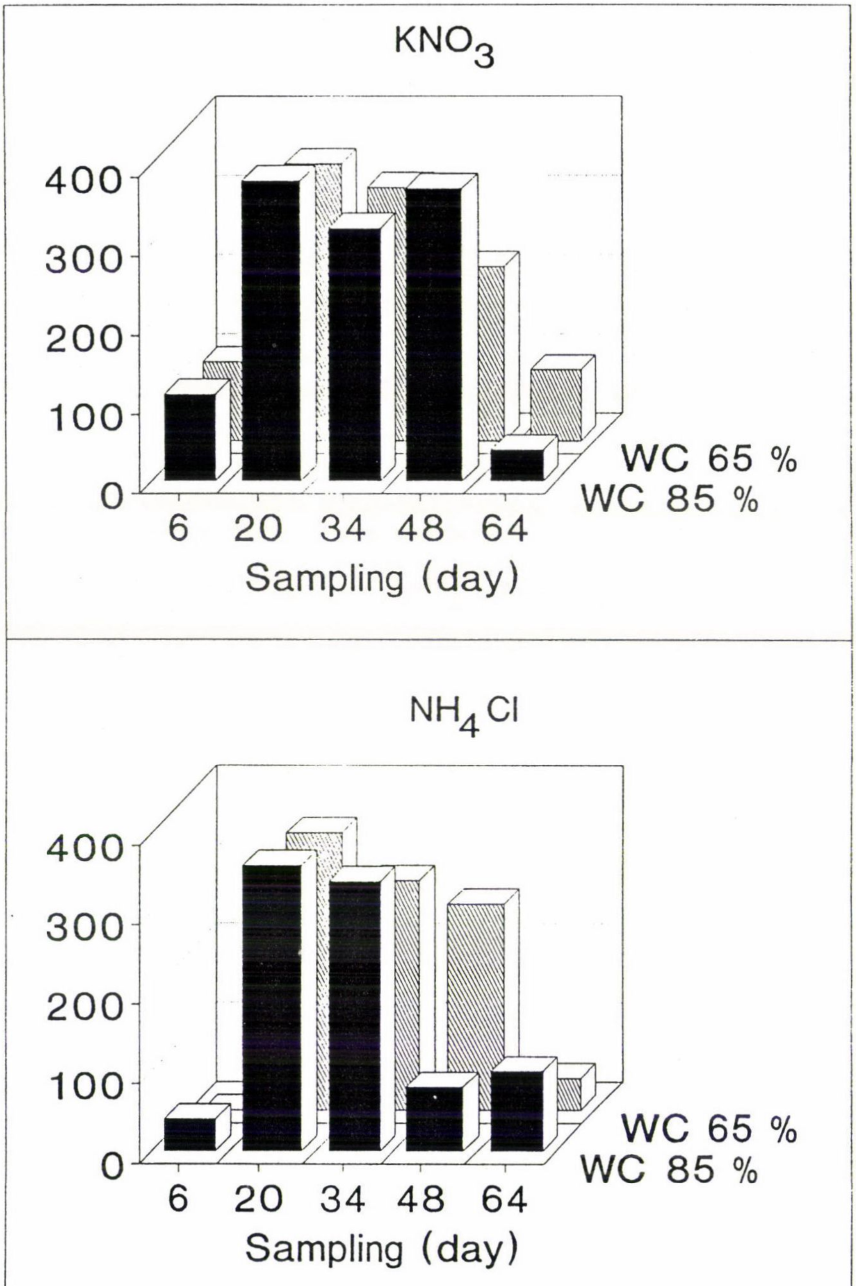

Fig. 4. Corrected values (mg N/1.8 litre) of N-containing gases in the root zone as measured in gas traps in the 1992 experiment without plants

In terms of the active agent % of the N fertilizers used, the amount of denitrification gases in the KNO_3-treated soil was 11% under plants and 14.9% without plants, while in the NH_4Cl treatment the gaseous loss from fertilizer N was 7.9% and 15.5% respectively, during the 10-week model experiment.

Conclusions

The main observations after two experimental years are as follows:

- There were significant differences in the quantities and proportions of N-containing gases generated by fertilization in the N fertilizer and water supply treatments and in treatments with and without experimental crops. A steady state of N fertilizer transformation was reached during the 7th week.
- In the first three weeks a more intensive gas generation due to denitrification could be observed in the crop experiments. After three weeks, a strong evolution of gases began in the cropless soil.
- In the case of the crop experiment, the soil air contained significantly more N_2 gas in the nitrate-N treatment than after treatment with ammonium-N fertilizer, and even more in the soil without a crop.
- In the case of fertilizer treatments the cumulative gaseous losses corresponded to 10–18%.

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N-CYCLE STUDIES IN A SOIL CORE INCUBATION EXPERIMENT

E. NÓTÁS[1], Á. BÁLINT[1], G. HELTAI[1], Z. TARR[1] and S. TÍMÁRI[2]

[1]DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY; [2]DEPARTMENT OF MICROBIOLOGY, GÖDÖLLŐ UNIVERSITY OF AGRICULTURAL SCIENCES, GÖDÖLLŐ, H–2103, HUNGARY

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The mobilization and immobilization processes of $^{15}NH_4{}^{15}NO_3$ were studied in a laboratory soil core incubation model experiment by means of the ^{15}N tracer technique. Brown forest soil was filled into 40 cm high plastic tubes and incubated for 30 days at 27°C at different moisture levels. The soil columns were divided into 10 cm segments and sampled on the 10th, 20th and 30th days. Total carbon content, different N forms of 1 M KCl-extractable NH_4^+ and NO_3^-, mineralizable nitrogen and total nitrogen were determined in each segment of the control and treated soil samples. A low C/N ratio (< 20) was calculated from the data of total carbon and total nitrogen for all soil samples. The recovery percentage of fertilizer N in different N forms was calculated from the $^{15}N/^{14}N$ isotopic ratio measurements. Extreme differences (6–50%) were found between mineral and mineralizable forms of fertilizer N at different moisture levels. A relatively rapid development of the stationary state in mineral N forms was observed during the first ten days. The level of mineral and mineralizable forms of available nitrogen was found to be highly influenced by the moisture level in the soil.

Key words: ^{15}N tracer technique, N transformation, N fertilizer mobilization, N fertilizer immobilization, recovery of N fertilizer

Introduction

The major part of the soil N content is found in different organic forms. By contrast the mineral forms of nitrogen (NH_4^+, NO_3^-), which are directly available for plants, are present only at low concentrations. Obviously, intensive plant cultivation requires a high rate of mineral nitrogen fertilizers, but this may cause environmental difficulties. On the other hand, plants often utilize directly only 50% or less of the applied nitrogen (Allison, 1966), while the remainder is immobilized either chemically or biologically, or is lost from the soil-plant system by leaching or denitrification processes. The transformation processes of nitrogen fertilizers are influenced to a large extent by microorganisms, whose energy source stems from the oxidation of organic materials. With an increase in temperature, soil moisture and oxygen supplies, the rate of degradation of organic materials increases. Mineralization and the soil moisture content are in strong positive correlation (Stanford and Epstein, 1974). It was also proved by Győri (1984) that the ammonium and nitrate contents of airdry soil did not change substantially, but with an increase in soil moisture both values showed an increase. The moisture content, frost, drying up and the carbon content of the soil influence the fixation of ammonium ions. The transformation and benefit of nitrogen fertilizers were investigated in pot and field experiments (Debreczeni, 1973). In the first year of the pot experiment 60% of the nitrogen content of the plant originated from fertilizers, while in the field this value was 40–50%. It was also shown that the transformation of fertilizer nitrogen in soil covered by plants is quicker in pots (3–4 weeks) than in the field (5–6 weeks). In a study of the fate of nitrogen fertilizer (75–150 kg/ha) over a three-year period, a loss of 46.93% to 74.53% was observed using the ^{15}N tracer technique (Zhifen and Guoging, 1990). In contrast, in glasshouse and gas-lysimetric experiments (Craswell and Martin, 1975) a very low loss of fertilizer nitrogen (ammonium and nitrate labelled with stable isotope ^{15}N) was observed. Ammonium and nitrate were almost completely recovered (97–99%) by soil and plant analysis over a period of 14–15 weeks.

A correct estimation of the nutrient-supplying capacity therefore requires a dynamic study of the N content available for plants, as influenced by the soil microbiological activity. The aim of the present paper was to study dynamic changes in the nutrient-supplying capacity of the soil after $^{15}NH_4{}^{15}NO_3$ application at different soil moisture contents.

Materials and methods

In the experiment a brown forest soil from Gödöllő (Hungary) was used. This soil is an acidic (pH: 5.4) cambic soil with low humus content (1.21%). The air-dried soil was moistened to 25, 50 or 75% of the water-holding capacity.

The soil was filled into PVC tubes (40 cm high and 3.6 cm in diameter). These tubes are similar to those used in the examination of the migration of nitrogen fertilizer by Varga and Beczner (1975). $^{15}NH_4{}^{15}NO_3$ fertilizer solution containing 10 atom% ^{15}N, corresponding to a fertilizer dose of 120 kg N/ha (24.4 mg N/column), was injected into the upper 3–4 cm layer. Half the soil cores were treated with fertilizer labelled with ^{15}N and the others were used as controls. Three parallel series were prepared. The soil cores were incubated for 30 days at 27°C at different moisture levels (25, 50 and 75% WHC). The columns were divided into 10 cm segments and samples were taken on the 10th, 20th and 30th days of the incubation period.

Analytical methods

The concentrations of the different N forms were determined in each segment of the control and treated soil samples.

Ammonium nitrogen and nitrate nitrogen were analysed by extraction with 1 M potassium chloride followed by distillation and titration using a modified Bremner (1965) method as described by Heltai et al. (1995).

Mineralizable nitrogen content was determined by treating the soil with 0.5 N sulphuric acid, followed by reduction and digestion using the Parnass-Wagner distillation and titration techniques (Heltai et al., 1995).

Total nitrogen content was analysed by Kjeldahl digestion with CrO_3, cc. H_2SO_4, followed by Parnass-Wagner distillation and titration as described by Tyurin (Ballenegger and Di Gleria, 1962).

The $^{15}N/^{14}N$ isotopic ratio of each N form was determined in each segment of the treated soil samples. The isotopic analysis was performed by the emission spectrometric method using an NOI-6 PC (Meier and Mauerberger, 1982).

The total carbon content was determined in each segment of the control and treated soil samples using a C/N analyser (NA 1500 Series 2, Carlo Erba). The C/N ratio was calculated from the total nitrogen and total carbon contents, as described by Nelson and Sommers (1982).

Table 1

ANOVA I, showing the significance of the differences between NH_4-N, NO_3-N, mineralizable N content and $^{15}N/^{14}N$ isotopic ratio in different nitrogen forms, carbon content and C/N ratio, and the soil core depth, soil moisture level and sampling time.

Source of significance	Between NH_4^+-N content		Between $^{15}N/^{14}N$ isotopic ratio in NH_4^+-N content	Between NO_3-N content		Between $^{15}N/^{14}N$ isotopic ratio in NO_3-N content	Between mineralizable N content		Between $^{15}N/^{14}N$ isotopic ratio in mineralizable N content	Between carbon content		Between C/N ratio	
	C	T	T	C	T	T	C	T	T	C	T	C	T
A: soil core depth	ns	s	s	s	s	s	ns	s	s	ns	ns	ns	ns
$LSD_{5\%}$	0.49	0.51	0.37	0.10	0.28	0.28	0.51	1.34	0.18	0.03	0.04	0.63	1.09
B: moisture level	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns
$LSD_{5\%}$	0.42	0.45	0.32	0.09	0.24	0.19	0.44	1.16	0.15	0.03	0.04	0.55	0.94
C: sampling time	s	s	s	s	s	s	s	s	s	s	s	s	s
$LSD_{5\%}$	0.49	0.51	0.37	0.1	0.28	0.22	0.51	1.34	0.18	0.03	0.04	0.63	1.09

Statistical significance (s) was considered at P=5% (ns - not significant). Least significant differences (LSD) at P=5% are given for each comparison. (C=control soil, T=treated soil).

Table 2

ANOVA II

For symbols and statistics see Table 1 (except: factor B is fertilizer treatment instead of soil moisture level

Source of significance	Between NH_4^+-N content			Between NO_3^--N content			Between mineralizable N content			Between carbon content			Between C/N ratio		
	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC
A: soil core depth	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	s
$LSD_{5\%}$	0.83	0.20	0.56	0.25	0.31	0.26	1.17	0.81	1.41	0.07	0.03	0.03	0.96	1.25	0.99
B: treatment	ns	s	s	s	s	s	s	s	s	ns	s	ns	ns	ns	s
$LSD_{5\%}$	0.58	0.15	0.40	0.17	0.22	0.18	0.83	0.57	1.00	0.05	0.02	0.02	0.68	0.89	0.70
C: sampling time	s	s	s	s	s	s	ns	s	ns	s	s	s	s	s	s
$LSD_{5\%}$	0.83	0.21	0.56	0.24	0.31	0.26	1.17	0.81	1.41	0.07	0.03	0.03	0.96	1.25	0.99

Statistical methods

The results were analysed by means of analysis of variance (ANOVA) according to Sváb (1981). Correlations were studied between the quantities of different nitrogen forms, the carbon content, the C/N ratio, the soil core depth (factor "A": 0–10, 10–20, 20–30 and 30–40 cm), the soil moisture level (factor "B": 25, 50 and 75% WHC) and the sampling time (factor "C": 0, 10, 20 and 30 days) (Table 1). Analysis of variance was performed again at constant soil moisture level. The differences between control and treated soil samples were studied. Factors "A" and "C" were the same as in the first analysis of variance, but factor "B" was the fertilizer treatment instead of the soil moisture level (factor "B": control and treated soil core) (Table 2).

Results

Changes in mineral N forms

In the control soil samples the amount of 1 M KCl-extractable mineral nitrogen (NH_4^+ and NO_3^-) showed an increase during the first 10 days at all moisture levels (Fig. 1). The rate of this increase was lowest at 50% WHC (3.3 times higher than the starting value). At 75% WHC, the rate was 5.5 times higher, and at 25% WHC it was 6 times higher than the starting value. After 10 days the values measured remained practically constant. The increase in the amount of mineral nitrogen was probably caused by microbiological activity due to the effect of moistening the soil.

In the treated soil samples the amount of 1 M KCl-extractable NH_4^+ and NO_3^- changed in different ways during the first 10 days; at 50% WHC it decreased rapidly to 50% of the starting value, at 25% WHC it decreased slightly, and at 75% WHC it showed a slight increase (Fig. 1).

After the 10th day, the amount of 1 M KCl-extractable nitrogen remained practically constant at all moisture levels. On the basis of these results it can be concluded that after the application of $^{15}NH_4{}^{15}NO_3$ fertilizer a stationary state of mineral nitrogen forms was established during the first ten days. The level of mineral N forms was found to be highly influenced by the soil moisture.

Changes in mineralizable N forms

In the control soil samples the mineralizable N forms increased slightly between the 0th and 30th days at 50% WHC, but did not change practically at 25% and 75% WHC (Fig. 1). At 50% WHC the curve reached a minimum on the 20th day.

In the treated soil samples the amount of mineralizable N forms decreased during the first 10 days at all moisture levels, the minimum value being measured at 50% WHC (Fig. 1). Between the 10th and 30th days it showed an increasing trend at 50% WHC. At 25% and 75% WHC it did not change substantially.

The amount of mineralizable N was compared with the amount of mineral N (Fig. 1) and differences were found depending on moisture level; at 50% WHC the amount of mineralizable N was 4–5 times higher than the mineral N amount in the

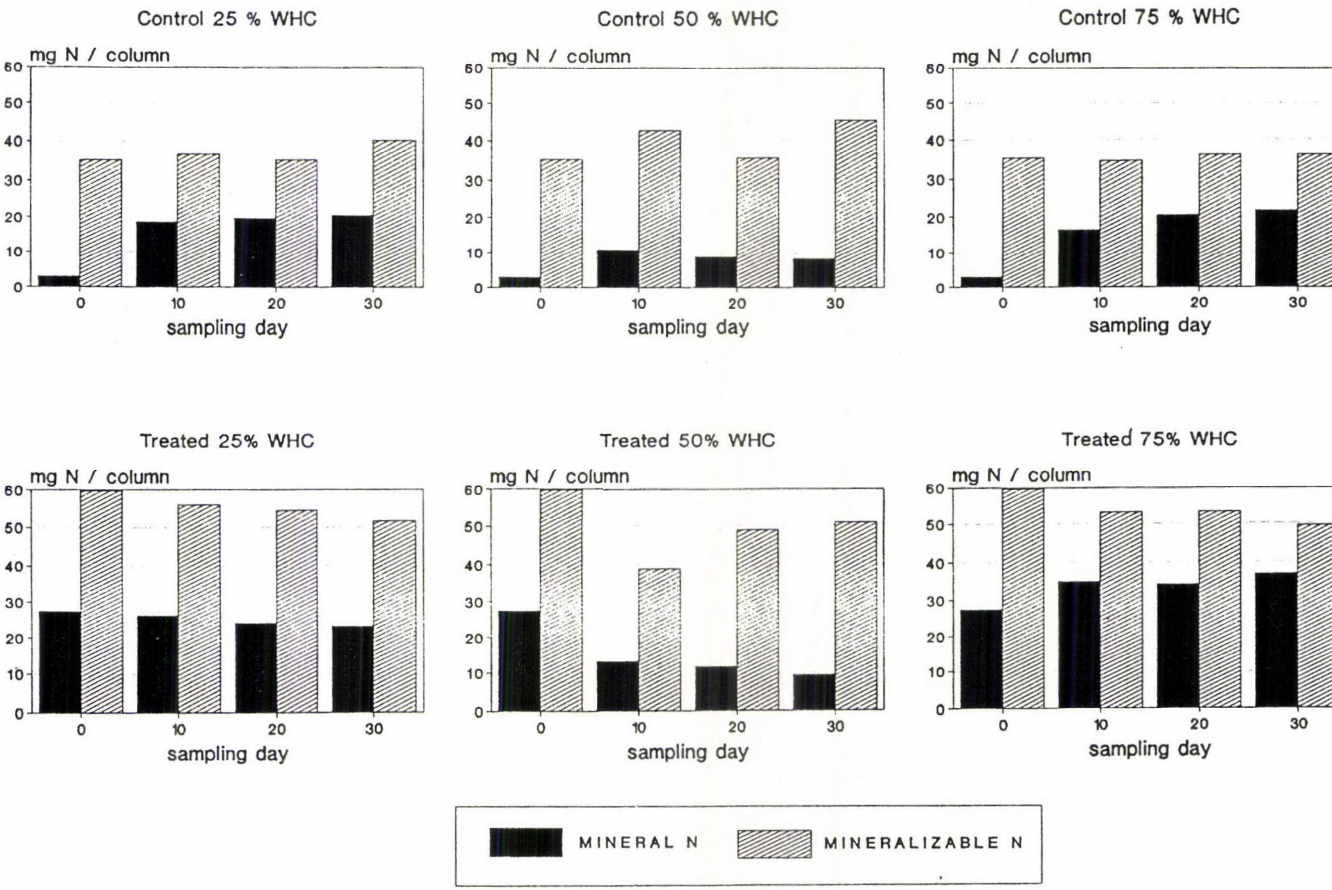

Fig. 1. Comparison of mineral and mineralizable N contents

Table 3
Changes in total nitrogen and carbon contents in control soils

Time (day)	Depth (cm)	N (mg/100 g soil) (Kjeldahl)			C (g/100 g soil)			C/N		
		25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC
0	0–10	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	10–20	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	20–30	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	30–40	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	Sum total	338.80	338.80	338.80	3.40	3.40	3.40	–	–	–
10	0–10	112.75	116.37	99.93	1.38	1.31	1.35	12.26	11.30	13.59
	10–20	126.00	111.46	85.93	1.28	1.37	1.41	10.23	12.34	16.48
	20–30	115.00	102.93	103.77	1.37	1.36	1.39	11.92	13.30	13.49
	30–40	108.75	107.86	113.65	1.36	1.37	1.40	12.51	12.75	12.39
	Sum total	462.50	438.62	403.28	5.39	5.41	5.55	–	–	–
20	0–10	88.94	102.46	105.17	1.42	1.36	1.35	16.14	13.32	12.90
	10–20	102.47	104.27	105.52	1.30	1.35	1.43	12.72	13.24	13.61
	20–30	100.69	101.12	108.76	1.32	1.36	1.40	13.11	13.45	12.93
	30–40	110.50	106.95	105.61	1.48	1.21	1.35	13.44	11.35	12.78
	Sum total	402.60	414.80	425.06	5.52	5.28	5.53	–	–	–
30	0–10	102.45	106.49	102.89	1.30	1.37	1.33	12.68	12.89	13.02
	10–20	102.92	98.90	92.19	1.37	1.35	1.38	13.38	13.71	14.98
	20–30	96.66	99.34	105.61	1.40	1.32	1.33	14.50	13.35	12.72
	30–40	116.35	104.72	100.33	1.28	1.37	1.41	11.07	13.17	14.06
	Sum total	418.38	409.45	401.02	5.35	5.41	5.45	–	–	–
$LSD_{5\%}$			15.90			0.11			2.19	

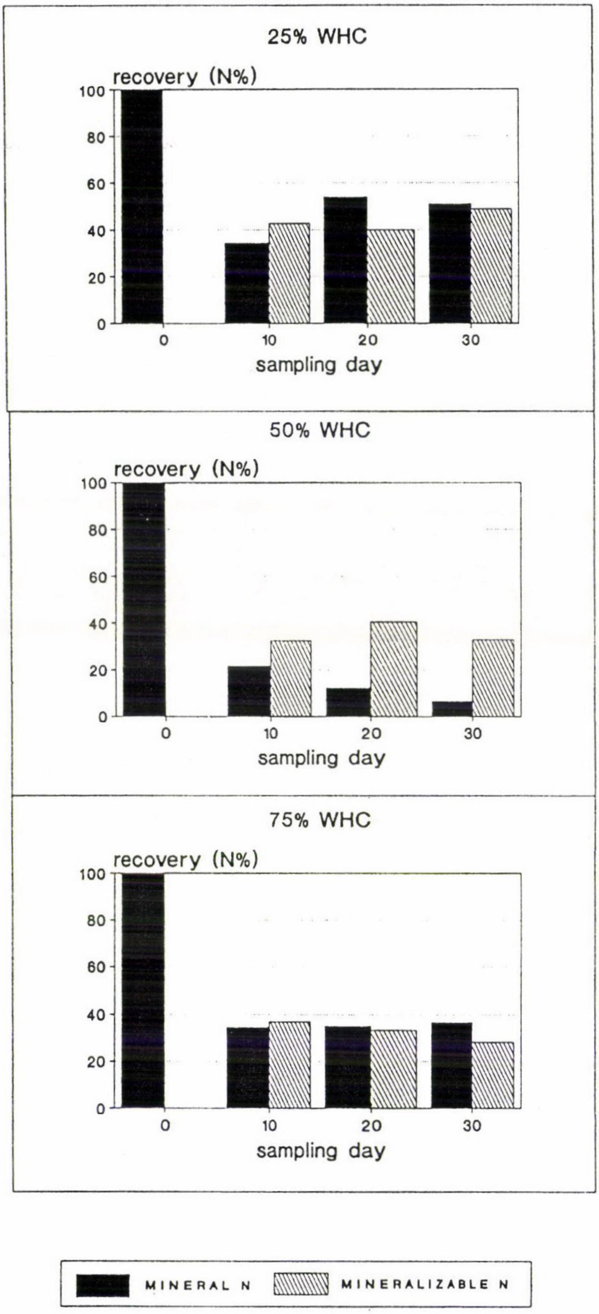

Fig. 2. Recovery % of fertilizer N in different N forms in soil cores

case of the control, while in the treated soil samples it was 3–5 times higher. At 25% WHC this difference was lower: mineralizable N was only twice as great as mineral N in both the control and the treated soil. At 75% WHC the difference was the smallest both in the control and the treated soil. On the basis of our results it can be assumed that the 50% soil moisture level is the most advantageous for N immobilization.

Changes in the total N content

In the control soil samples the total N amount increased significantly during the first 10 days at all moisture levels. The rate of this increase was maximum at 25% WHC (Table 3). Between the 10th and 20th days the total N content increased slightly at 75% WHC, decreased rapidly at 25% WHC and decreased slightly at 50% WHC. In the last 10 days the total N amount increased slightly at 25% WHC, but decreased at the other moisture levels (50% and 75%).

In the treated soil samples the change in the total N was similar to that in the control soil during the first 10 days. It was maximum at 75% WHC. Between the 10th and 20th days the trend showed a decrease at 75% WHC in contrast with the other moisture levels (Table 4). In the last 10 days an increase in total N was observed at all moisture levels.

Recovery of fertilizer N in different N forms

Figure 2 shows the recovery of fertilizer N content in different N forms (mineral and mineralizable N). The N injected on the 0th day was regarded as 100%. Substantial differences were observed at different moisture levels for the fertilizer N recovered in mineral and mineralizable forms. On the 30th day these amounts were approximately 50% and 50%, respectively at 25% moisture level, 6% and 35% at 50% WHC and 35% and 30% at 75% WHC.

On the basis of these results it can be concluded that after the 10th day there is no significant change in the recovery of fertilizer N.

Distribution of the recovery of fertilizer N in mineral form as a function of time and depth

The distribution of the recovery of fertilizer N in mineral form was studied as a function of time and of the depth of the soil column. Figure 3 illustrates the amount of injected N (100%) and the percentage of mineral N recovered. Significant quantities could not be measured in the 10–40 cm segments in the columns.

Change of C/N ratio in soil core

As the transformation of nitrogen forms is influenced by the carbon content of the soil, the total carbon content of all soil samples was determined and the C/N ratio

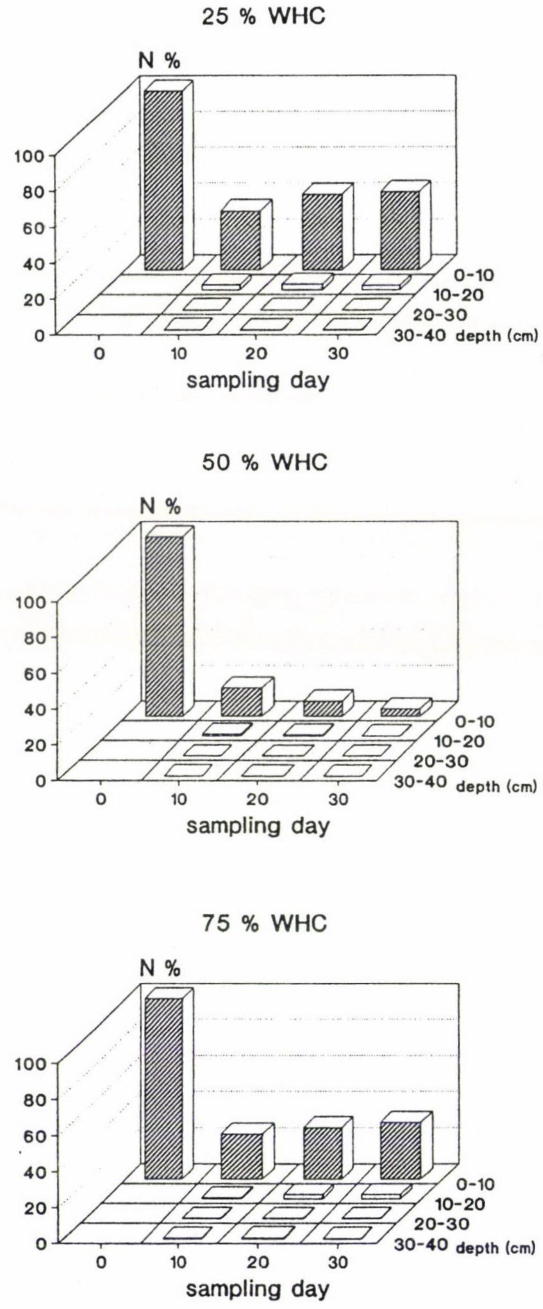

Fig. 3. Distribution of the recovery % of fertilizer N in mineral nitrogen form as a function of time and depth

Table 4

Changes in total nitrogen and carbon contents in treated soils

Time (day)	Depth (cm)	N(mg/100g soil) (Kjeldahl)			C (g/100g soil)			C/N		
		25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC	25% WHC	50% WHC	75% WHC
0	0–10	109.11	109.11	109.11	0.85	0.85	0.85	7.79	7.79	7.79
	10–20	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	20–30	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	30–40	84.70	84.70	84.70	0.85	0.85	0.85	10.00	10.00	10.00
	Sum total	363.21	363.21	363.21	3.40	3.40	3.40	–	–	–
10	0–10	100.69	117.70	118.15	1.34	1.36	1.44	13.32	13.57	12.27
	10–20	98.45	108.30	121.72	1.72	1.36	1.42	17.47	14.52	11.70
	20–30	114.12	108.75	106.96	1.37	1.44	1.45	11.64	14.66	14.12
	30–40	103.38	101.14	117.70	1.48	1.41	1.41	14.32	16.46	11.99
	Sum total	416.64	435.89	464.53	5.91	5.57		–	–	–
20	0–10	102.04	117.70	111.43	1.38	1.35	1.37	13.79	11.60	12.56
	10–20	108.30	114.12	90.403	1.32	1.36	1.37	12.24	13.84	15.36
	20–30	113.22	115.01	112.77	1.29	1.38	1.29	11.39	11.17	11.64
	30–40	98.90	115.91	118.15	1.31	1.42	1.40	13.32	13.18	12.01
	Sum total	422.46	462.74	432.75	5.30	5.51		–	–	–
30	0–10	105.17	107.41	121.28	1.33	1.35	1.30	12.85	12.91	10.90
	10–20	113.22	122.17	111.43	1.30	1.35	1.41	11.67	9.73	12.71
	20–30	124.86	122.17	108.30	1.34	1.38	1.36	10.73	15.12	12.54
	30–40	112.77	107.70	112.78	1.34	1.37	1.35	11.92	12.23	12.00
	Sum total	456.02	466.32	453.79	5.31	5.45	5.42	–	–	–
$LDS_{5\%}$			31.71			0.15			3.78	

was calculated (Tables 3 and 4). The C/N ratio ranged from 10–16 in the control soil samples and from 8–17 in the treated soil samples. This low C/N ratio (< 20) is favourable to the mineralization processes (Stefanovits, 1992), as confirmed by the present results: an increase in the mineral nitrogen content was experienced during the experiment in all soil samples except treated soil at 50% WHC. On the basis of the above results it can be supposed that at the 50% soil moisture level the mineral nitrogen was transformed into organic form.

Discussion

The present results have confirmed that even in the case of the same fertilizer treatment the nutrient-supplying capacity of the same soil changes within extremely wide limits depending on the moisture level and temperature, due probably to the microbiological activity of the soil. It has also been proved that only a low concentration of mineral fertilizer nitrogen remains directly available to the plants, while the remainder is chemically or biologically immobilized. On the basis of our results, a soil moisture content of 50% WHC is the most advantageous for nitrogen immobilization. When comparing the mineral nitrogen contents of the soil layers (10 cm segments), significant quantities of N fertilizer were only recovered from the upper 10 cm layer of the soil core. The same results were observed in a cylinder lysimeter for non-irrigated soils by Németh et al. (1989). It was also shown in the present study that in the lower soil layers only a very small amount of mineral nitrogen can be detected, and this did not change either in time or in depth, which may be due to the very slow rate of diffusion in the soil core. On the basis of these results it can be concluded that rapid N transformation occurs during the first 10 days, when a stationary state was formed at all moisture levels in both control and treated soil samples, in accordance with results reported by Korenkov (1968) and Reid (1969). In the interests of economical and environmentally friendly fertilizer application it is important to know how long a mineral form of N will remain available to the plants, so an increase in the sampling frequency is proposed. On the other hand, it is also important to study the N transformation during a longer period than 30 days in order to estimate the seasonal changes occurring over the whole vegetation period.

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EFFECT OF GROWTH MEDIUM ON THE GROWTH OF CEREALS IN THE PHYTOTRON

T. TISCHNER[1], K. RAJKAINÉ VÉGH[2] and B. KŐSZEGI[1]

[1]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, MARTONVÁSÁR, HUNGARY
[2]RESEARCH INSTITUTE FOR SOIL SCIENCE AND AGRICULTURAL CHEMISTRY OF THE HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST, HUNGARY

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The effect of the growth medium composition was studied in the case of wheat and barley plants grown in phytotron units. Four soils were created by mixing chernozem with the commercial composts Vegasca and Terra-vita and with sand. The major data of these soils were as follows: bulk density (g $\cdot cm^{-3}$) 1.47, 1.39, 1.30 and 1.39; pH 7.67, 7.52, 7.39 and 7.64; organic matter content (%) 2.93, 3.42, 5.72 and 2.99; AL-P_2O_5(mg.kg^{-1}) 352, 369, 507, 643; AL-K_2O (mg kg^{-1}) 612, 598, 706, 1428. At the end of the experiment measurements were made on a number of morphological traits (height, tiller number, leaf number) and yield parameters (spike number, grain number, grain mass). It could be concluded from the results that a 1/5 ratio of compost and 1/5 ratio of sand in the soil used for phytotron experiments is sufficient from the point of view of both soil structure and nutrient content.

Key words: controlled environment, growth medium composition, phytotron, plant growth, Terra-vita, Vegasca

Introduction

One of the greatest advantages of phytotronic plant research over field experiments is that the environmental conditions under which the experimental plants grow and develop can be programmed to the values desired by the researcher and are thus reproducible. At the present level of technical development, the major parameters of the plant environment, such as air temperature and humidity, atmospheric carbon dioxide concentration, and the intensity, spectrum and duration of illumination, can be adjusted to the desired values under artificial conditions with relative ease. However, when experiments are set up using natural soil, it is difficult, if not impossible, to achieve "programmability and reproducibility" for the growth medium or to optimise plant growth as regards various soil components. In experiments on nutrient uptake the plants are raised in hydroponics or on artificial soils (e.g. perlite, vermiculite, etc.) and watered with nutrient solution (Downs, 1975; Downs and Hellmers, 1975; Berry, 1978; Bernáth et al., 1982). The effect of soil components on plant growth under artificial conditions has not yet been given much attention, and the experiments reported were mostly conducted in greenhouses (Mastalerz, 1977; Hanan et al., 1978).

Many factors determining the root environment are different in pots from those existing in the field, including the soil temperature, the rate of drying out, the moisture distribution in the root zone, the soil volume available for rooting and the availability of the nutrients. These factors are greatly influenced by the size of the pot (Townend and Dickinson, 1995).

Under small pot experimental conditions the spatial extension of the root system is substantially inhibited, resulting in high root density, which may, for example, be in excess of 3 $cm \cdot cm^{-3}$ by the 4th week of vegetative development in the case of barley (Végh, 1991). When nutrients are transported to the roots in considerable quantities by mass flow (e.g. nitrate) the deficiency zones around the roots may overlap each other within a short time, which means that neighbouring roots must compete with each other. Due to the limited soil volume available and the inhibition of root growth, there may be a reduction in the uptake of less mobile nutrients, present in the soil solution at low concentrations and readily adsorbed on the soil, such as phosphorus and potassium. The replenishment of water and absorbed nutrients may become a critical factor within a very short space of time.

The surface of contact between the roots and the soil particles is considerably greater in pots, where the soil is disturbed, than in the field, where the roots generally grow into the macropores. Although this increase in the surface of contact means an increase in the availability of water and nutrients, there is also a greater danger of inadequate oxygen supplies, especially when large quantities of water are added. In a pot experiment, Kooistra et al. (1992) recorded a 27% increase in root-soil contact, while porosity dropped by 16%. If oxygen supplies are inadequate there will be a reduction in water and nutrient uptake and hormone production in the roots, while the development of the shoots will be retarded.

For most of the experiments set up in the Martonvásár phytotron natural soil is used, as this accords with the aim of the experiments (Tischner, 1981). Due to the restricted size of the pots, however, the quantity of soil available to each plant under phytotronic conditions is only a fraction of that available in the field. For example, the raising of wheat from seed to seed is carried out in plastic pots with a volume of 1.5 litres. For other plant species a similarly restricted quantity of soil is available for each plant (10 litres for maize and 7 litres for sunflower). It is thus necessary to enrich the nutrient content of the soil. The soil brought from the institute nurseries, a chernozem with forest residues, is thus mixed for the purposes of most experiments with commercial peat and compost mixtures containing fertiliser (originally Florasca B, and more recently Vegasca III).

In order to replace the macro- and microelements extracted from the soil in the course of experiments which may last as long as six months, the plants are regularly watered each week with nutrient solution (complex NPK fertiliser, Volldünger, Wuxal). Due to the limited amount of soil and the drying effect of the 0.3 m/s air flow, the plants need watering every day. In order to avoid excessive soil compaction due to daily watering the soil is always mixed with 20% sand.

In order to achieve further improvements in the quality of phytotron experiments, studies were made on soil containing various quantities of different types of nutrient additives.

Materials and methods

The experiments were set up in a PGR-15 (Conviron) plant growth cabinet in the phytotron of the Agricultural Research Institute of the Hungarian Academy of Sciences. The climatic programme employed (designated "t21-ny21") was a modified version of the "t2-ny2" spring-summer programme (Tischner et al., 1997) routinely used in the Martonvásár phytotron.The test plants were the spring wheat Lona and the spring barley Orbit, with 10 plants per treatment, grown in 18 cm tall plastic pots with a volume of 1.5 litres. In the course of the vegetation period the plants were watered on 13 occasions with Wuxal (2ml/l), 11 times with Volldünger (2g/l) and 4 times with a complex fertiliser (NPK; 5g/l) solution.

The four soil mixtures used in the experiment are listed in Table 1.

Table 1
Composition of the soil mixtures

Treatment	Chernozem	Vegasca	Terra-vita	Sand
I	4	–	–	1
II	3	1	–	1
III	2	2	–	1
IV	3	–	1	1

The soil chemical and soil physical analyses of the soil mixtures were carried out following the recommendations in the Manual of Soil and Agrochemical Analysis (Búzás, 1988; 1993). The data were evaluated using variance analysis (Sváb, 1973).

Results and discussion

The major data of the soil analysis, carried out in the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, are summarised in Tables 2 and 3.

The addition of Vegasca caused a slight reduction in the pH of the soil mixtures. A double quantity of Vegasca (treatment III) increased the humus content to twice that of the control. Substantial differences were found in the AL-extractable P and K contents, characteristic of the available phosphorus and potassium reserves of the soil mixtures. The available phosphorus content of the mixture containing Terra-vita was almost double and the potassium content more than double that of the control or treatment II.

The bulk density and particle size distribution of the soil mixtures were extremely similar; the quantity of the physical clay fraction (<10 mm) was practically the same in all four mixtures.

Table 2
Data of the soil mixtures used in the various treatments

Treatment	Bulk density ($g\ cm^{-3}$)	$pH(H_2O)$	Organic matter content (%)	$AL\text{-}P_2O_5$ ($mg\ kg^{-1}$)	$AL\text{-}K_2O$ ($mg\ kg^{-1}$)
I	1.47	7.67	2.93	352	612
II	1.39	7.52	3.42	369	598
III	1.30	7.39	5.72	507	706
IV	1.39	7.64	2.99	643	1428

The soil water retention characteristic (SWRC) curves indicative of the water retention ability of the soil, which provide information on the water : air ratio in the soil at various moisture contents, are presented in Figure 1.

The course of the SWRC curves is similar for all four treatments, but compared to treatment I, those of treatments II and IV show a slight shift towards the higher moisture content range, while this shift is much greater for treatment III. As can be seen from the figure, treatment III led to an increase in water retention ability throughout the range studied. The moisture content of the soil mixture was 8–10% higher than that of the control at all water potential values, which, considering the correlation between water potential and water conductance, is advantageous as regards both water and nutrient transport to the roots and surface evaporation. Greater pore volume leads to a higher degree of aeration and a better oxygen supply to the roots. However, the water content available to the plants (approx. 13% in all treatments) did not change when the additives were mixed into the soil.

Higher moisture content and/or greater available P or K content in the soil facilitates a greater rate of diffusion, thus extending the P and K replenishment zone around the roots. A larger soil mass thus participates in plant nutrition than in the case of drier soil or soil with a lower content of available P or K (Végh et al., 1990).

Table 3
Particle size distribution of the soil mixtures used in the various treatments

Treatment	Particle size (%)						
	> 250 μm	250–50 μm	50–20 μm	20–10 μm	10–5 μm	5–2 μm	<2 μm
I	52.7	25.1	6.6	1.8	2.2	2.4	9.3
II	56.6	20.5	6.9	2.6	1.4	2.7	9.4
III	62.4	14.6	8.2	2.0	1.2	2.2	9.4
IV	57.3	20.6	6.9	1.9	2.9	1.2	9.1

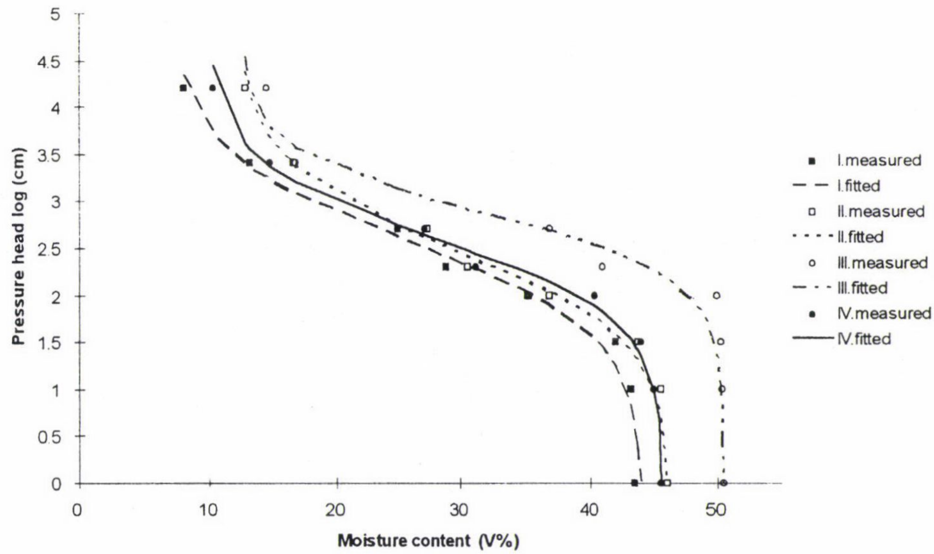

Fig. 1. The SWRC curves of the soil mixtures

In small pots used in lengthy experiments the magnitude of the available phosphorus reserves becomes a particularly critical factor, since high root density, competition for nitrate and water and the limited quantity of soil available to the roots, mean that compensatory root growth is unable to make sufficient phosphorus available to the plant.

The morphological (plant height, leaf number, etc.) and yield (spike number, seed number, etc.) data recorded at the end of the experiment are presented in Table 4. Compared to the results obtained for the chernozem and sand mixture used as a control (treatment I), no significant differences were found in the morphological traits of either wheat or barley in treatment II, and only in one or two cases in treatments III and IV. The situation was quite different for the yield parameters, where there was only one case when there was no significant difference compared to the control.

When setting up the experiment one important question was whether an increase in the quantity of compost (Vegasca) in the soil mixtures used for phytotronic plant growth would provide better conditions for the plants and thus lead to improved yield components. The results indicate that no significant difference can be found between treatments II and III for any trait, while a comparison of the same quantity of different composts (Vegasca and Terra-vita) presents a more ambiguous picture. In the case of wheat, for instance, the grain number and grain mass were significantly higher in treatment IV than in treatment II. For barley, too, all the yield components were better in treatment IV, even if the differences were not significant.

Table 4
Means of quantitative data for Orbit and Lona under different soil conditions

Treatment	Plant height (cm)	No. of shoots/plant	No. of leaves/plant	No. of spikes/plant	No. of seeds/plant	Total seed weight/plant (g)
			Orbit			
I	71.6	9.6	37.5	6.4	153	8.45
II	73.4	10.1	41.1	8.6*	213*	11.35*
III	74.0*	9.9	41.1	7.5	206*	10.96*
IV	72.1	12.1*	48.4*	8.9*	238*	12.87*
*$LSD_{5\%}$	1.96	1.43	4.97	1.61	44.4	2.20
			Lona			
I	58.3	8.8	34.3	2.8	85	4.29
II	57.3	9.1	35.0	4.1*	166*	7.28*
III	58.2	11.1*	38.8*	4.0*	175*	7.62*
IV	58.9	9.4	35.6	4.3*	204*	8.68*
*$LSD_{5\%}$	1.1	1.15	3.55	0.63	27.3	1.13

The results lead to the conclusion that a 1/5 ratio of compost and 1/5 ratio of sand in the soil used for phytotron experiments is sufficient from the point of view of both soil structure and nutrient content. The nutrients extracted by the plants in the course of the experiment both can and must be replaced by watering with nutrient solutions (NPK fertiliser, microelements). It can also be concluded from the soil physical and soil chemical parameters and from the yield data that under the given experimental conditions the quantity of available phosphorus, which is required by the plants in large quantities during reproductive development, must be given special attention when compiling growth media, since in such a relatively small soil volume, even with satisfactory water supplies and weekly nutrient replacement, the availability of phosphorus, which is readily adsorbed on the soil and thus not very mobile, may become a limiting factor.

The results also drew attention to the fact that when preparing soil mixtures, instead of increasing the quantity of compost, greater attention should be paid to the choice of composts with various compositions now available on the market.

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Review

POSSIBILITIES OF MERINO IMPROVEMENT AND BREEDING INTEGRATION IN CENTRAL EUROPE AND MIDDLE ASIA

L. VERESS[1], R. NIZNIKOVSKI[2] and K. MEDEBEUKOV[3]

[1] UNIVERSITY OF AGRICULTURAL SCIENCES, DEBRECEN, HUNGARY, H–4015, P.O. BOX 36
[2] FACULTY OF ANIMAL BREEDING, WARSAW UNIVERSITY OF AGRICULTURAL SCIENCES, 02–793 BRWINÓW, POLAND
[3] ANIMAL RESEARCH INSTITUTE, ALMA-ATA, KAZAKHSTAN

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In the regions in question Merino breeding and improvement has a long-standing tradition, and wool-prolific and mutton types of Merinos are bred in these areas. The meat-producing capacity of wool-meat type Merinos can be increased by 10–20% when crossing them with European and Asian meat types. The halfbred ewes produced by crossing the local Merinos with those carrying Fec^BFec^B genes can be lambed every 8 months, and the number of progeny can be increased by 0.6–1.0 lambs with each lambing, while fine-wool output of the same quality can also be achieved. By means of three breed crosses, with the application of Asian fat-tailed breeds as terminal partners, the meat production per ewe can be doubled, and the meat output and meat quality of slaughter lambs can be significantly improved. To achieve these aims, a new union and federal structure should be worked out to protect and support breeding activity. A Foundation for Animal Improvement should be brought into being to finance the extra expenses of improvement and animal-breeding production, and to elaborate the mutual interests of breeding integration .

Key words: sheep improvement, crosses, gene Fec^B, breeding integration

The constant renewal of the Merino

The first Merino stock arrived in Saxony, Central Europe from Spain in 1765 where it became acclimatized and was developed into the "Electoral" type. The first sheep breeding association in Europe was founded in 1814, near Brno in the Austrian Empire and the effectiveness of this association was proved by the "Negretti" type produced in this area. The long-wooled Russian "Mazajev" type was developed somewhat later. In the middle of the 19th century a new wool processing technology stimulated Merino breeders to modernize the breeding line to the "Rambouillet" type. From the middle of the 19th century the drop in wool prices and the increase in the price of mutton encouraged breeders to develop the "Merino Precoce" type in France and the Mutton Merino in Germany. The registration of both types dates back to 1928.

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After the 1st World War the "Askania Nova" Animal Breeding Research Institute was founded in the Soviet Union. From here good Rambouillet rams and their sperm were air-freighted to Eastern Europe and to several territories of Middle Asia, where carpet-wooled breeds were bred, and where the climate seemed favourable for Merino breeding. Between 1926 and 1971 22 new Merino breeds were registered, and the Mutton Merino spread throughout Eastern Europe and Middle Asia (Litovtsenko and Esaulov, 1977). Up till the restructuring of the Soviet Union, 60% of all the sheep stock were Merino breeds. After the 2nd World War the proportion of Merino types quickly increased in Bulgaria and Rumania as well, and in Hungary 98% of all the ewe stock were Merinos.

The Booroola Merino, living in Australia, was found to have a major gene stimulating the ovulation activity of the ovary. The inheritance of the ovulation rate proved to be intermediate; litter size, however, showed partial dominance (Bindon et al., 1982; Bindon, 1984; Davis and Kelly, 1983). This finding, which was of great economic importance, significantly changed the methods used to increase prolificacy in sheep improvement (Turner, 1978; Clarke, 1982; Bradford, 1985). Booroola Merinos were first brought to Hungary – and to Europe - from Australia and New Zealand in 1980 at the proposal of one of the present authors (Veress), and this was followed by further imports in 1984 and 1986. On the basis of a prolification rate of 160–167% for 1535 ewes originating from the second crossed generation of Hungarian and Booroola Merinos, this new breed was registered in 1992 under the name "Prolific Merino"; at present there are about 3000 ewes of this type registered in the flockbook.

Czechoslovakia bought Booroola Merinos from New Zealand in 1985 (Horák, 1988), Germany bought them from Hungary (Debrecen) in 1989, and Poland also bought them from the Haldon Station in New Zealand (Czlonkowska et al., 1991).

As a result, several countries of Central Europe now have wool-meat and prolific types of Merinos, whereas in Eastern Europe and Middle Asia the wool-meat and meat-wool types of Merinos are bred. We would like to talk about how improvement and breeding integration could and should comply with the potential effects of the political, economic and market relations, which have undergone significant changes in this large region in recent years.

The situation prior to the changes

In the Soviet Union, sheep farming, which was carried on mostly in unfavourable agricultural conditions, used to be supported in such a way that the internal state purchase price of greasy wool was 6–10 times more than its price on the world market. This system of government subsidies was maintained up till 1990 in the socialist block, except Hungary. Besides the numerous advantages, however, negative effects started to come to the surface, as follows:

- numerous valuable – but not wool-type – local breeds, like Romanov, Gissar, Edelbayev, Sharadzin, etc., were pushed into the background,
- as a consequence of the "quantitative" attitude which prevailed for a long time, in places where Merinos had originally become well adapted, they were often replaced by crossbred wool-producing types,
- the strong wool orientation hindered the specialization of certain Merino breeds, the development of prolificacy, and the capacity of milk and meat production,
- the development of new breeds was the chief aim and commercial breeds were rarely crossed even in places where market-oriented production was practised.

In the last decade of the Soviet Union, the Australian Merino Medium was bred in the East European region and the Australian Merino Strong and Pollwarth Breeds in the territories east of the Urals, partly to standardize the numerous breed variants, and partly to achieve a simultaneous improvement in staple length, coat density and rendement.

In the ex-socialist countries there have been several research initiatives during the past decade aimed at a more up-to-date improvement of Merino breed variants and the more profitable utilization of the breeds.

By lambing Polish Merinos every 8 months Korman et al. (1986) increased litter size by 28% as compared to that of a single lambing. Veress and Kakuk (1976) worked out a method for the early weaning of lambs at 30–40 days of age, and for fast fattening, which resulted in the rearing of Merino slaughter lambs to weights of 30–35 kg at 100–150 days of age. For those applying this method, pelleted whole feed for raising lambs was manufactured and made available. Early weaning age was considered as one of the main conditions required for frequent lambing, which is becoming more and more widespread in field practice (Veress et al., 1988).

In the German Democratic Republic (GDR), König (1978, 1979) crossed Mutton Merino (MM) and Merino Landrace (ML) ewes with Finnish × Friesian F_1 rams, and both the Merino breeds and the halfbred ewes were lambed every 8 months with the help of a "lighting programme". The annual litter size of the halfbred ewes ranged between 2.72–2.82, thus exceeding the annual prolificacy of pure-bred Merinos by 0.8–1.4 lambs; the mortality rate of suckling lambs was 23% (Süss and König, 1987). In Poland, too, several research stations were set up to utilize the possibilities for yield increase latent in "three-breed" crossing. Veress (1986) tried to increase the inclination for frequent lambing, prolificacy, and milk-producing capacity by cross-breeding Hungarian Merinos with the Romanov type. Borys and Osikowski (1992) tried to improve the prematurity and prolificacy of Polish Merinos by cross-breeding with the Finnish Landrace type.

In the GDR Göhler (1978) used Mutton Merinos, while in Poland Osikowski and Borys (1976), Jankowski and Niznikowski (1985) and Kozal and

Slosarz (1986) used Polish Merinos for cross-breeding with different European meat-breeds, in order to find out which breeds could be combined the most favourably. The best daily weight gain and muscularity were achieved by crossing the following genotypes: Merino × Suffolk, Merino × Texel, and Merino × Berrichon du Cher.

The majority of the governments, however, continued to give preference to the wool production types, to lambing Merinos once a year, and to pure breeding. Breeding integration in the Soviet Union, and also in other countries of the socialist block, remained mostly twofold. In pedigree flocks and provisional pedigree flocks flock-book registration was carried out, but the proportion of these was not more than 10% of the given type. Pure breeding was mostly performed in the remaining – productional – part of the flock, and rams could be obtained only from pedigree flocks. The possibilities offered by utility breed crossings could be used, though to a negligible extent, in the GDR and Poland.

Changes recommended for improvement

The closed internal market regulated by a planned economy in this region collapsed during 1989 and 1990; the long international economic crisis also had a dramatic effect on prices, and the number of Merinos dropped. There were two previous Western European patterns which could be followed to change the utilization and breed composition of the sheep flock:

1. In France and Germany Rambouillet Merinos were gradually replaced by meat breeds since the end of the 19th century.
2. In Great Britain a very rational and economical breeding integration was gradually built up, starting from the turn of the century. Though the present ewe population is made up of 70 breeds, only 53% of the ewes are pure-bred, 45% of which belong to 10 carpet-wooled mountain breeds. The remaining 47% of the ewe stock are made up of almost 300 crossbred variants, mainly of the carpet-wooled mountain type and the prolific longwool type, the majority of which can still be classed in 10 cross types, which are mated with "terminal" mutton breeds, such as Suffolk, Hampshire Down, Texel, Charolais, etc. Forty percent of the whole ewe population can be found in hill or upland sheep farms, where the utilized progeny is about 95%; 17% of the ewes are kept in flocks in uplands, where the number of weaned lambs is about 130%. Forty-three percent of the ewes are kept in lowlands, where the utilized progeny is about 145%. Sixty-nine percent of the slaughter lambs are the cross-products of terminal meat breeds (Croston and Pollott, 1994).

In the future, especially in Central Europe, but in Eastern Europe and Middle Asia as well, it would be expedient to exploit the opportunity offered by cross-breeding to a much greater extent.

In compliance with this, the ewe stock in the region can be grouped into three categories as regards utilization:

1. Meat-wool type and wool-meat type Merinos, which are well adapted to the given ecological circumstances, and which at the same time can be used as starting partners in market-oriented crossbreedings.
2. Merinos carrying Fec^B genes, which could be used as "B" partners in utility breed crossings. Cross-breedings carried out with these types have a great advantage, since the body size and wool production of halfbred ewes hardly differs from those of local Merinos.
3. "Terminal" mutton breeds, which are used for cross-breeding in Western Europe, and Middle-Asian fat-tailed breeds with big bodies and rapid growing capacity (Edelbayev, Dzaidara, Gissar, Sharadzin, etc.), the meat of which is delicious, and which play an important role in providing fat for the local population.

The adaptability, endurance and excellent wool-producing abilities of breeds belonging to the first category are worth preserving. It is worth considering whether it is possible to improve their rutting and conception ability, prolificacy and milk-producing ability throughout the year, when fed on the available forage, and whether it is possible to try frequent lambing with them. The future of Mutton Merinos presents a serious dilemma, since these breeds are much more demanding as regards forage, and they are not competitive with types like Suffolk, Texel and Berrichon du Cher for any of the following characteristics: muscularity, daily weight gain during the fattening period, slaughtering and forage utilization.

The Fec^BFec^B genotypes of Booroola Merinos and Prolific Merinos can be grouped in the second category. The Prolific Merinos, including these breeds as well, are often rightfully criticized for the fact that it is risky to bring up lambs from a large litter and that their fattening performance is weak.

However, these breeds have the following very valuable characteristics: compared to normal Merinos, their rutting and conception ability throughout the year is more favourable, as is their genetic inclination to frequent lambing (Bindon et al., 1982). The heritability of these characteristics proved to be average for both the Hungarian Merinos ($h^2 = 0.28 \pm 0.08$) and the Prolific Merinos ($h^2 = 0.38 \pm 0.08$), and selection for these characteristics proved to be very successful (Burgkart et al., 1986; Veress et al., 1993).

One of two populations carrying Fec^B genes was lambed once a year, whereas the other was frequently lambed. As a result of frequent lambing, ovulation rate and litter size significantly decreased (Table 1). With frequently lambed Prolific Merinos, the economically unfavourable litter size of 4–5 was

Table 1
Ovulation rate (OR) and litter size for lambing

Genotype	$Fec^B Fec^B$		$Fec^B Fec^+$		Reference
	OR	Litter size	OR	Litter size	
Booroola Merino	4.60	2.56	3.18	2.39	Dodds et al., 1991
Prolific Merino	4.98	2.07	2.50	1.73	1995/1996, Debrecen

considerably reduced as a result of the frequent lambing method (Fig. 1), and although the proportion of genotypes carrying Fec^BFec^B genes increased in 1993 as compared to 1992, there was no increase in the litter size. These types are valuable for their compensational ability as regards growth, and for the fact that their sexual dimorphism is greater than that of other Merino breeds (Veress, 1991). In the nucleus flock of Prolific Merinos in Debrecen, for example, we can simultaneously increase the proportion of Fec^BFec^B genotypes and the mature weight, and improve the inclination to frequent lambing, the staple length and the fibre diameter. Though these characteristics are not in positive genetic correlation with each other, there still tends to be a significant development in each feature from generation to generation. When improving these breeds priority must be given in selection to the weight gain of young lambs, intensive growth and increased mature live weight.

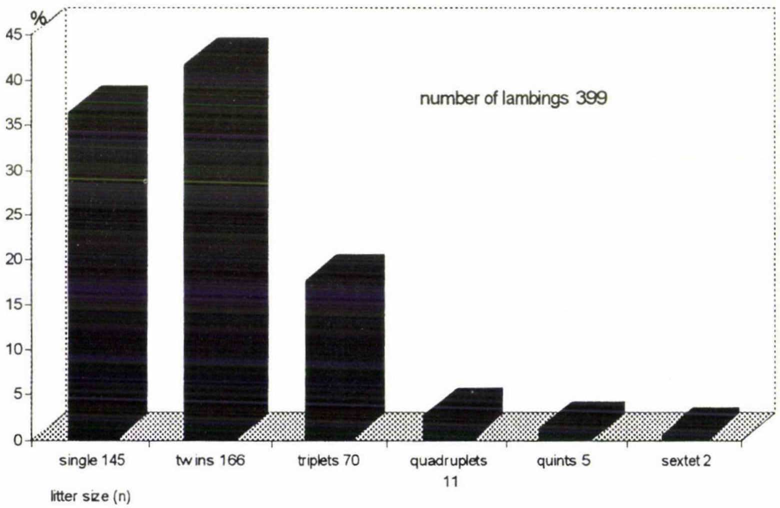

Fig. 1. Distribution of litter size (%) for Prolific Merino (Debrecen, 1995/1996)

The weight of halfbred ewes must not decrease significantly when crossing them with basic-type Merinos. The achievement of an increase in mature live weight, however, seems to be a somewhat simpler genetic task. The method for improving types by two-breed or three-breed mutton utility crossings has already been well established, and a vast amount of international literature is available on the topic, so it will not be discussed here.

As for the future of Middle Asian local types (Edelbayev, Dzaidara, Gissar, Sharadzin), however, the situation is completely different. Nucleus flocks of these breeds should be gathered from types which are outstanding for following characteristics:

– exceedingly large weight
– good muscularity
– abundant milking capacity
– possible capacity for mutton-fat deposition.

Further selection should then be concentrated on fast growing intensity and capacity, to achieve a more favourable slaughtering percentage and forage utilization, and possibly to achieve a more favourable fatty acid composition in the mutton fat. Rams produced in these stock-farms could be progeny tested by the rules of recurrent selection; the one that produces the best crossing result should be favoured in purebreeding.

Breeding integration recommendations

In Central and Eastern Europe, and in Middle Asia as well, it is worth assessing in each country what proportion of the Merino ewe stock is needed for a sufficient production of ewe lambs to ensure an annual 20–25% selection and generation change. After this, it would be advisable to crossbreed the remaining ewe stock with the previously mentioned mutton types, to aim at a better utilization of the heterosis effects produced by the crossings. Once this has been achieved, the sheep farms producing for the market could be divided into two groups, as follows: pure-breed multiplication could be carried out in the first group, whereas the second group would deal exclusively with crossing. The possible variations of breeding integration are outlined in Figure 2. In this way 5 or 6 breeding methods could be developed here, similarly to Great Britain and other countries of the British Commonwealth, where sheep breeding is highly developed.

The more differentiated the breeding integration is with respect to the final product, the bigger the yields and the more profitable the results which can be expected. The introduction of frequent lambing could further increase these results. According to Lengyel (1989) the weight gain of the utilized lambs from

Hungarian Merino × Booroola F_1 ewes and Suffolk rams exceeded that of purebred lambs from Hungarian Merino ewes by 40% at the age of 120 days, and the mutton quality of the slaughter lambs also improved at the same time.

Several conditions will be required if a more complex structure for breeding integration is to be introduced. These are as follows:

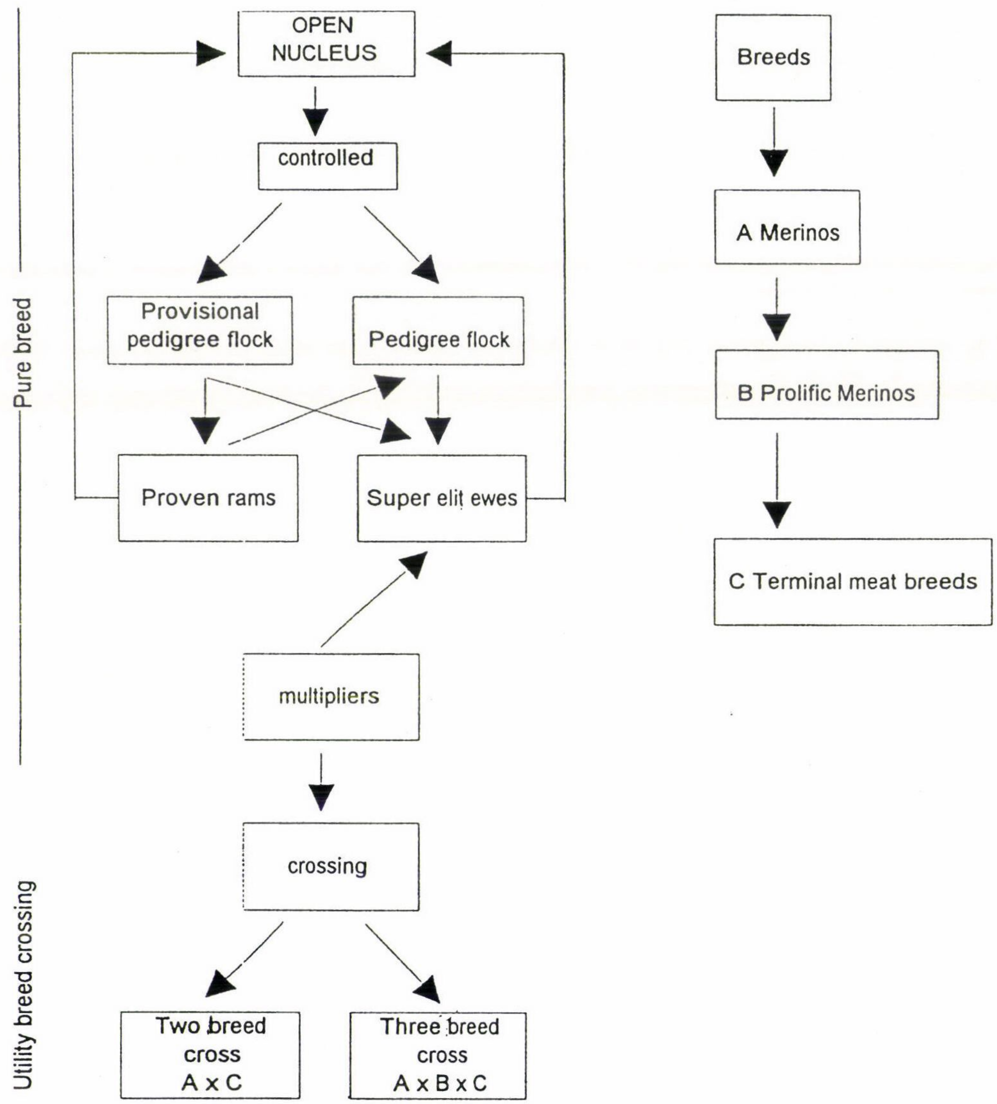

Fig. 2. A possible structure for breeding integration

– an Animal Improvement Foundation should be brought into being based on 0.5% of the proceeds of all product sales. This Animal Improvement Foundation is designed to cover the extra expenses of improvement and flockbook registration, subsidies for animals to be bred domestically, and the expenses of progeny testing.

This foundation should be supported both by the sheep trade and by the sheep processing industry:

– breeders should elect from among themselves suitable leaders for the regional and national associations. The task of these associations is partly to provide production security for breeders and to maintain an acceptable profit margin, and partly to check the validity of breeding data and to supervise breeding discipline and hygiene;
– the date of purchase or sale and the stage of development of the animals marketed should be registered in contracts made within or between the breeding associations. Such complex breeding integration can be developed only on the basis of mutual confidence.

Because of the above-mentioned risks and worries, the introduction of three-breed utility breed crossings and of lambing ewes every 8 months is advisable only in regions and sheep farms where the energy and protein demands of the considerably larger forage basis (twice as much as usual) necessary for this type of farming can be provided. These feeding requirements are well described in a detailed survey published by Robinson et al. (1983).

Sheep possess a prolification capacity considerably greater than that of other ruminants. This biological capacity in the field of slaughter production should be utilized primarily in places where there is a definite consumption demand. Merinos are suitable for the doubling of present slaughter lamb yields per ewe in a more up-to-date breeding integration.

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Book review

J. Horváth (ed.): *Diseases of Field Crops.* Mezőgazda Kiadó, Budapest, 1995. 328 pp. ISBN 963 7362 21 5

It is a pleasure to see this major reference work, edited by Professor J. Horváth of Pannon Agricultural University, Keszthely. In this source book the authors, all long-experienced in teaching, aim to provide a large quantity of diverse information related to the pathogens of field crops and the diseases they cause. As pointed out in the foreword, the appearance of this volume should meet a need felt by students at Hungarian agricultural universities and colleges for decades. In fact, the authors of this book have been successful in gathering and writing up all the basic and updated knowledge of diseases of any kind occurring in agricultural fields, both on a large scale and on small acreages.

With no introduction, the book comprises chapters on the major field crops (wheat, barley, rye, oat, maize, sunflower, potato, sugarbeet, lucerne and clover), as well as some minor crops (rice, oilseed-rape, tobacco, linseed, peas, soyabean, Phaseolus bean, Faba beans and poppy) commonly grown in Hungary. In each chapter a similar sequence is followed to include descriptions of diseases caused by viruses, bacteria and fungi. For a few crops, physiological disorders or phytoplasma vs. spiroplasma diseases are also treated.

The book is well illustrated: the majority of disease symptoms and of plant pathogens are shown in original drawings, photographs and micrographs making it easier for the reader to identify both the pathogens and diseases in question. For those requiring more detailed information on a particular subject, an extensive list of bibliography and another list of references for further reading are included in the book. For students, it was a good idea on the part of the authors to list the major scientific journals (either Hungarian or international) and periodicals dealing with field crop diseases. The sources of some of the figures, and a general subject index close this comprehensive, well-written volume. I do believe "Diseases of Field Crops" is a significant contribution to the higher education strategy in agriculture, and it will assist not only students and teachers but also agronomists and all others associated with field crop production and management.

F. VIRÁNYI

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THE ROLE OF PHOTOSYNTHESIS IN HARDENING TO FROST OF WINTER WHEAT AND RYE

T. I. TRUNOVA, S. V. KLIMOV, N. V. ASTACHOVA, G. S. KARASEV and G. V. NOVITSKAYA

TIMIRYAZEV INSTITUTE OF PLANT PHYSIOLOGY, RUSSIAN ACADEMY OF SCIENCES
UL.BOTANICHESKAYA 35, MOSCOW, 127276 RUSSIA

Seedlings of winter wheat and winter rye were hardened at 2° C under illuminance of 2.5 klx with a 16h photoperiod for 10 days. At the beginning of hardening, the ratio of light-saturated gross photosynthesis to dark respiration was 40% higher in the rye than in the wheat plants. By the end of hardening, this ratio was increased two to three times in both crops, but the difference between the crops remained valid. We believe that an increase during hardening in the indexes (sugar, lipid and cytoplasmic protein content in the leaf tissues, the ratios of high mol wt polypeptides to those of low mol wt, of membrane lipids to membrane proteins, of phospholipids to sterols, of unsaturated to saturated fatty acids, and of plastoglobule area to the chloroplast area), can be considered in terms of a creation of additional sinks at various levels of plant structural organization, which maintain relatively high rates of photosynthesis under low temperatures. In rye plants, a higher total sink capacity provides better frost adaptation than in wheat plants.

Key words: *Secale cereale*, *Triticum aestivum*, frost resistance, photosynthesis, sink-source relations

Introduction

Winter cereals use the energy-rich organic products of low-temperature photosynthesis for the functional and structural rearrangements of acclimating cells (Tumanov, 1979). A negative correlation between the plant growth rate in autumn and its capacity for acclimation is common within a single plant species. A comparison between rye and wheat plants showed that rye plants developed higher frost resistance combined with fairly active autumnal growth.

In this work, we tried to explain the difference in rye and wheat frost resistance in terms of alterations in sink-source interrelations occurring at different levels of plant structural organization.

Materials and methods

Seedlings of winter rye (*Secale cereale* L., cv. Voskhod 2) and winter wheat (*Triticum aestivum* L., cv. Mironovskaya 808) were used.

The plants were grown in a 1/4 Knop solution at 18°C under illuminance of 10 klx with a 16h photoperiod for seven days. Cold hardening was performed in a climatic chamber at 2°C under illuminance of 2.5 klx with a 16h photoperiod for 10 days. This stage was a period of maximum CO_2 assimilation by the first leaf, making up 80% of the total plant photosynthesis. Eight-day-old untreated plants and 17-day-old hardened plants at the same developmental stage, namely second leaf emergence, were analysed.

The rates of apparent CO_2 assimilation at light saturation conditions and of dark respiration were measured using a Uras 2T infrared gas analyser (Hartmann und Braun, Germany), as described earlier (Davydenko et al., 1992). Gross CO_2 assimilation was estimated as a sum of apparent CO_2 assimilation and the CO_2 that evolved after the light was switched off.

The techniques for extraction, separation and quantitative estimation of lipids, fatty acids and proteins and the methods for ultrastructural studies of cells and chloroplasts, including morphometric measurements, were described in Klimov et al. (1993), while sugar extraction and quantitative estimation were performed according to Davydenko et al. (1992).

Table 1 represents the means of three to four biological replicates (each in three to four analytical recordings) and their standard errors. Only differences significant at P = 95% are taken into account.

Table 1

The effect of cold hardening on photosynthesis and respiration activity (mg CO_2 g dry wt^{-1} h^{-1}) at different measurement temperatures, the ratio of gross photosynthesis to respiration, the content of sugars, proteins and lipids (percentage of leaf dry weight), high mol wt/low mol wt polypeptides (100–232 kD)/(14–40 kD), membrane lipids to membrane proteins, phospholipids (mol. wt 725–856) to sterols (mol. wt 415–684), unsaturated to saturated (U/S) fatty acids, and the ultrastructure of chloroplasts

Index	Wheat		Rye	
	Non-hardened	Hardened	Non-hardened	Hardened
Apparent CO_2 assimilation				
18° C	11.0±1.5	4.3±0.4	17.3±1.5	12.1±0.4
2° C	6.2±0.7	3.1±0.1	9.2±0.9	8.7±0.1
Dark respiration				
18° C	8.2±1.8	4.1±0.4	6.2±0.5	4.0±0.4
2° C	1.2±0.1	0.6±0.1	1.5±0.1	1.3±0.3
Gross photosynthesis / dark respiration ratio				
18° C	2.6	2.1	4.0	3.4
2° C	5.3	6.8	7.5	8.4
Percentage of dry weight:				
Sugars	8.9±1.0	21.2±1.3	8.9±1.0	29.7±1.4
Proteins	3.0±0.1	6.7±0.4	2.5±0.1	5.8±0.5
Lipids	0.8±0.2	2.6±0.2	1.5±0.2	3.5±0.2
Ratios:				
High mol wt/low mol wt polypeptides	0.18	0.57	0.25	1.26
Membrane lipids/membrane proteins	1.03	1.22	1.32	1.72
Phospholipids/sterols	0.46	1.50	1.95	7.89
U/S fatty acids	4.75	5.68	5.73	6.67
Percentage of the total area of the chloroplast section:				
Stroma	81.4	92.2	82.4	87.7
Grana	13.6	6.3	12.2	7.9
Starch grains	4.0	0.02	4.0	2.4
Plastoglobules	1.0	1.5	1.4	2.0

Results and discussion

Low temperature markedly changed plant gas exchange, influencing both photosynthesis and dark respiration (Table 1). Respiration was inhibited more severely than photosynthesis. For example, a comparison of hardened plants at 2° C with non-hardened plants at 18°C showed that the respiration in hardened plants was inhibited to a greater extent than photosynthesis.

The photosynthetic component of gas exchange in both hardened and non-hardened rye seedlings prevailed to a greater extent than in wheat seedlings. In non-hardened rye at a measurement temperature of 18° C, the ratio of gross photosynthesis to dark respiration was equal to 4.0, whereas it was only 2.6 in the wheat plants. Hardening shifted these ratios at measurement temperature of 2°C to 6.8 and 8.4 for the wheat and rye plants, respectively.

The ratio of gross photosynthesis to dark respiration in the whole plant is assumed to be equal to the source-to-sink ratio. Thus, the source-to-sink ratio is shifted more towards the source in both hardened and non-hardened rye plants, as compared to wheat plants.

In the course of cold adaptation, a readjustment of the sink-source balance, which was disturbed by low temperature, occurred because of the appearance of additional sinks for assimilates at various levels of the plant structural organization. Cell elongation is more sensitive than cell division to falling temperature. As a result, the longitudinal growth of both wheat and rye seedlings was almost completely (90%) inhibited. Biomass production, however, continued to increase in both plant species. The contents of some biochemical constituents of biomass were changed. The estimation of sugars, proteins and lipids, i. e. the substances of crucial importance for plant survival under low-temperature stress, supported this conclusion. Hardening enhanced the accumulation of sugars, proteins and lipids 2–3-fold in both the cultures, but the contents of sugars and lipids were higher in winter rye plants.

According to the sink-source concept of plant adaptation to stress factors (Klimov et al., 1990), increased sink capacity (such as that in the case of rye plants) should result in qualitative changes in the products of photosynthesis, that is, in the accumulation of "material-consuming" and "energy-rich" substances with high glucose costs for their synthesis (Chiariello et al., 1989). Cold hardening caused the preferential accumulation of high mol wt cytoplasmic proteins. In the leaves of hardened wheat plants, the ratio of 100–232 kD to 14–40 kD proteins was increased 3-fold, and in the leaves of hardened rye plants it was increased 5-fold. Another index, the ratio of membrane lipids to membrane integral proteins in cells and chloroplasts, is of crucial importance for plant adaptation to stress factors. Following hardening, the relative content of lipids, the material-consuming compounds, was increased 1.2- to 1.3-fold. This increase was more pronounced in the rye membranes. The composition of membrane lipids was changed in favour of high mol wt compounds as well; following hardening, the ratio of phospholipids (with mol wts from 776 to 857) to sterol (with mol wts from 415 to 684) increased three- to four-fold. In the rye plants, this ratio increased to a somewhat greater extent than in wheat. The fatty acid composition of the lipids also changed during cold hardening as the ratio of more material-consuming and energy-rich unsaturated to saturated acids was increased.

Hardened wheat and rye plants differed somewhat in chloroplast ultrastructure. In the wheat plants, the grana filled 6.3% of the chloroplast section area; in the rye plants, the total granum area was slightly larger (7.9%). This evidence agrees with the higher photosynthetic capacity in rye leaves. In the hardened rye plants, the sum of the chloroplast grana, plastoglobules and starch grains filled 12.3% of the chloroplast section area, whereas they occupied only 7.8% of the area in the wheat plants.

We believe that these alterations "level off" the source-sink balance, which is disturbed by low temperature. All these alterations are a prerequisite for hardening. For example, the fluidity of the membrane lipid bilayer is increased and manifests itself as the preservation of membrane semipermeability at low temperatures. The increase in membrane fluidity, naturally, shifts adverse processes to lower temperature ranges. In non-hardened and hardened rye, as compared to wheat plants, the fluidity of the membrane lipid bilayer is likely to be more pronounced, as demonstrated by higher lipids/proteins, phospholipids/sterols and unsaturated/saturated fatty acids ratios.

Thus, a more perfect system of sinks for assimilates, exhibited at cellular, subcellular and membrane levels of rye structural organization, permits this crop to maintain a higher capacity for photosynthesis during cold hardening. As a result, significant reserves of metabolites, including membrane constituents, are produced, which are utilized as respiration substrates during subsequent overwintering and spring growth.

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EFFECT OF LOW TEMPERATURE ON NUCLEIC ACID SYNTHESIS IN WHEAT AND BARLEY

E. PÁLDI, G. SZALAI and T. JANDA

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, P.O.BOX 19, HUNGARY

In the course of the experiments studies were made on the effect of low temperature on the rRNA maturation process and on correlations between cold-induced changes and the cold tolerance of winter wheat and barley varieties. It was found that as the results of low temperature treatment quantitative changes took place in the rRNA maturation process. In the poorly frost-resistant line the accumulation of the last precursors (1.4 and 0.9×10^6 dalton) of the two stable cytoplasmic rRNAs (1.3 and 0.7×10^6 dalton) could be observed. In the second series of experiments the interaction between the genetically determined frost resistance and the quantity of 1.4 MD rRNA precursor was investigated for 18 wheat and barley varieties with various degrees of frost resistance. It was concluded from the results that the 1.4 MD rRNA precursor could always be demonstrated in genotypes with poor frost tolerance but not in varieties with good frost tolerance. A positive correlation was found between the quantity of precursor and the frost tolerance of the genotype. These observations suggest that, depending on the degree of frost tolerance, low temperature slows or inhibits the synthesis of cytoplasmic rRNA, and thus also that of ribosomes.

Key words: *Hordeum vulgare* L., *Triticum aestivum* L., cold treatment, frost tolerance, maturation process, ribosomes, rRNA precursors, wheat, barley

Abbreviations: MD: million dalton; rRNA: ribosomal ribonucleic acid; DNA: dezoxyribonucleic acid; LT: lethal temperature; P_i: inorganic phosphate; cpm: counts per minute; PAS: sodium 4-aminosalicylate; SDS: sodium dodecyl sulphate

Introduction

The physiological and biochemical processes which lead to cold tolerance, or the adaptation of plants to low temperature, are extremely complex. The molecular and genetic mechanisms which regulate these processes are not sufficiently well known. In recent years, however, more and more information has been reported, for example on the interaction between cold effect and protein synthesis, the relationship between cold effect and soluble protein composition, the mapping of genes related to frost resistance, results in the field of carbohydrates and enzyme adaptation, etc. (Perras and Sarhan, 1989; Sutka, 1994; Guy et al., 1992; Galiba et al., 1995; Veisz et al., 1996).

The effect of low temperature on the nucleic acid metabolism has long been the subject of research in Martonvásár. In the course of this work it has been demonstrated that cold-induced rRNA synthesis takes place in seedlings as the result of low temperature during the first few days of cold treatment. It was established that the extent of cold-induced rRNA synthesis is closely correlated to the rRNA cistron number (Dévay and Páldi, 1977; Páldi and Dévay, 1977; Páldi and Dévay, 1983). Results obtained in other fields showed that low temperature leads to changes in the minor base composition of the cytoplasmic RNAs, on the fluorescence induction parameters and even in the quantity of polyamines (Janda et al., 1994; Rácz et al., 1996).

The aims of the present research were: 1) to determine whether any changes were induced by low temperature in the rRNA maturation process, and if so, 2) to analyse these changes and to discover if they were correlated with the frost resistance of wheat and barley varieties.

Materials and methods

Plant material

The two winter wheat lines used in the experiments were derived from a cross between the varieties Bezostaya 1 and Moisson after several years of positive and negative selection for frost resistance. Mv 11-75 possesses good frost resistance, while that of Mv 13-74 is poor. In addition, seven winter wheat varieties with various degrees of frost resistance (Cheyenne, Bezostaya 1, Mironovskaya 808, Bánkuti 1201, Libellula, Cappelle Desprez and Bersée), 3 spring wheats (Penjamo 62, Siete Cerros and Chinese Spring), 6 winter barley varieties with various degrees of frost resistance (Hohenturm, Mv 35, Mv 34, Béta 2 soros, Montpellier and Ager III) and 2 spring barleys (Mk 42 and Mk 47) were also tested.

RNA assay

The seeds were germinated under sterile conditions on 1% agar containing 2% sucrose at room temperature for 72 h in darkness. Etiolated 72-hour-old seedlings were exposed to cold treatment at 1°C for various lengths of time (3, 5 and 8 days). After the cold treatment the intact seedlings were incubated with their root tips resting in 5 ml 1000-fold diluted Knop solution per 50 seedlings, containing 0.5 mCi $^{32}P_i$, for 24 hr at 1°C.

The wheat and barley seedlings were homogenised in buffered sucrose medium (Loening, 1967) and the homogenate was centrifuged (1000 g, 5 min). SDS (0.5%) and PAS (5%, w/v) were added to the supernatant fraction and the suspensions were shaken with phenol containing 0.1% (w/v) 8-hydroxiquinoline at 4°C. The phases were separated by centrifugation at 1000 g for 10 minutes and the phenol extraction was repeated twice. The RNA was precipitated from the final supernatant by the addition of 2% (w/v) sodium acetate and 2.5 vol of ethanol at –20°C. The final RNA precipitate was reprecipitated twice from 0.3 M sodium acetate, washed once with ethanol and partially dried for a few minutes *in vacuo* (Kirby, 1965). DNA-free RNA, prepared according to Wells and Ingle (1970), was then dissolved in electrophoresis buffer containing 5% (w/v) sucrose to give a final concentration between 0.5 and 2.0 mg/ml. The purified RNA was fractionated by electrophoresis on 2.4% polyacrylamide gel (50 V for 3.5 h). The distribution of radioactivity was determined by freezing the gels in dry ice to scanned length prior to cutting 0.3 mm slices, which were dried on filter paper. The slices were counted in a liquid scintillation spectrometer (Loening, 1969).

Estimation of frost tolerance

For the purpose of the frost tests, seedlings of the various barley and wheat varieties were raised for two weeks in an earth-sand mixture (3:1). Freezing was carried out in programmed freezing chambers, where the temperature gradient of cooling and warming was 0.5°C an hour

(Tischner et al., 1997). In order to determine the LT_{50} value the 14-day-old seedlings were first hardened for 5 days at 2°C, after which the plants were divided into as many parts as there were freezing temperatures (–5, –10, –15, –18, –20 and –25°C). The seedlings were kept at the relevant freezing temperatures for 48 hours, then raised for a further 14 days at 15°C (16 h light, 8 h dark, 70% relative humidity) in a phytotron unit (PGV-36). The survival percentage was then determined as a function of the freezing temperatures applied. On the basis of the graphs obtained, the critical temperature was taken to be that at which 50% of the plants survived. The mean results of three biological replications were taken as the basis for determining the LT_{50} values.

Results and discussion

In order to study the rRNA maturation process, two wheat lines were chosen which differed, as the results of selection, chiefly as regard the degree of frost tolerance. These lines were Mv 11-75, which has good frost tolerance (LT_{50}: –16.8°C) and Mv 13-74, which has poor frost tolerance (LT_{50}: –3.1°C). In the course of this experiment, studies were made on the effect of cold induction on the relative proportions of rRNA precursors.

As could be seen from the data, as the result of cold treatment (for example after 5 days), the proportion of 2.9 MD/2.3 MD high molecular mass rRNA precursors in the line Mv 13-74 shifted in favour of the latter fraction, while the proportion of 1.4 MD/1.3 MD and 0.9 MD/0.7 MD precursors shifted in favour of the former fractions as treatment continued (Table 1). In the frost-tolerant line Mv 11-75 the 1.4 MD and 0.9 MD rRNA precursors could not be demonstrated during the treatment. The 2.9 MD/2.3 MD ratio was practically constant at a value above 2 (Table 2). The change in the relative proportion of precursors as cold treatment proceeded in the poorly frost-tolerant line suggests the retardation of the rRNA maturation process. By contrast, in the frost-tolerant line the absence of 1.4 MD and 0.9 MD precursors indicates the uninterrupted synthesis of stable 1.3 MD and 0.7 MD cytoplasmic rRNAs.

Table 1

Effect of low temperature on rRNA synthesis in the wheat line Mv 13-74 (LT_{50} = –3.1°C)

Length of cold treatment	Incorporation of $^{32}P_i$ into the cytoplasmic rRNAs and their precursors (cpm)					
	2.9 MD	2.3 MD	1.4 MD	1.3 MD	0.9 MD	0.7 MD
Control	13167	5981	–	24173	–	16822
3 days	11052	9168	4636	21002	3451	14143
5 days	7871	9095	14825	12964	13794	6075
8 days	7659	9877	20464	5598	14268	3699
$LSD_{5\%}$	850	442	1200	949	1092	813
$LSD_{1\%}$	1192	619	1682	1331	1531	1140

Notes: Molecular weights of stable cytoplasmic rRNAs are 1.3×10^6 and 0.7×10^6 dalton. Incorporation of $^{32}P_i$ into the different rRNA precursor at 1°C for 24 h. 50 µg total rRNA was separated by PAGE (2.4%, 50 V, 3.5h). LT_{50} = critical temperature characteristic of the actual frost hardiness of the wheat line. Data are the average of five experiments.

Table 2
Effect of low temperature on rRNA synthesis in the wheat line Mv 11-75 ($LT_{50} = -16.8$°C)

Length of cold treatment	Incorporation of $^{32}P_i$ into the cytoplasmic rRNAs and their precursors (cpm)					
	2.9 MD	2.3 MD	1.4 MD	1.3 MD	0.9 MD	0.7 MD
Control	13908	6558	–	26115	–	15644
3 days	12956	5766	–	22958	–	14173
5 days	12214	5658	–	21814	–	15006
8 days	11482	5059	–	20988	–	14319
$LSD_{5\%}$	1228	675	–	2367	–	1232
$LSD_{1\%}$	1722	947	–	3318	–	1728

Notes: see Table 1

In the second experiment detailed studies were made on changes in the quantity of 1.4 MD rRNA precursor in barley and wheat varieties with different degrees of frost tolerance. The proof of a correlation between the quantity of 1.4 MD rRNA precursor and frost tolerance was based on results obtained when analysing the rRNA maturation process. Previous results suggested that a positive correlation existed between the quantity of 1.4 MD rRNA precursor and the frost tolerance of wheat and barley genotypes characterised by LT_{50} values. The 1.4 MD rRNA precursor was chosen for the examinations as it is easily demonstrated. The results of experiments carried out on 10 wheat and 8 barley varieties with different degrees of frost tolerance are presented in Tables 3 and 4.

Table 3
The effect low temperature on the quantity of 1.4 MD rRNA precursor in different wheat varieties

Varieties	Incorporation of $^{32}P_i$ into the 1.4 MD rRNA precursor (cpm)	LT_{50} (°C)
Winter wheats		
Cheyenne	–	−18.4
Bezostaya 1	–	−16.2
Mironovskaya 808	–	−16.0
Bánkuti 1201	851	−12.8
Libellula	1768	−10.7
Cappelle Desprez	2062	−8.6
Bersée	3890	−4.8
Spring wheats		
Penjamo 62	5228	−4.8
Siete Cerros	6279	−3.0
Chinese Spring	6210	−2.9
$SD_{1\%}$	561	1.19
$SD_{5\%}$	407	0.86

Notes: Incorporation of $^{32}P_i$ into the 1.4 MD rRNA precursor at 1°C for 24 hr in the dark. LT_{50}= critical temperature characteristic of the actual frost hardiness (resistance) of the wheat cultivars. Data are the means of five experiments.

Table 4
The effect low temperature on the quantity of 1.4 MD rRNA precursor in different barley varieties

Varieties	Incorporation of $^{32}P_i$ into the 1.4 MD rRNA precursor (cpm)	LT_{50} (°C)
Winter barleys		
Hohenturm	–	–16.8
Martonvásári 35	–	–15.6
Martonvásári 34	–	–13.7
Béta 2 soros	710	–12.6
Montpellier	1469	–10.3
Ager III	1469	–10.3
Spring barleys		
Mk 42	4090	–6.3
Mk 47	4168	–6.0
$SD_{1\%}$	502	2.14
$SD_{5\%}$	362	1.54

Notes: Incorporation of $^{32}P_i$ into the 1.4 MD rRNA precursor at 1°C for 24 hr in the dark. LT_{50}= critical temperature characteristic of the actual frost hardiness (resistance) of the barley cultivars. Data are the means of five experiments.

It was found that in winter wheat varieties with good frost tolerance (Cheyenne, Bezostaya 1 and Mironovskaya 808) no 1.4 MD rRNA precursor could be demonstrated during incubation at 1°C with $^{32}P_i$. As the degree of frost tolerance, i.e. the LT_{50} value, decreased, the quantity of this precursor increased. This change was the most pronounced in spring varieties (Penjamo 62, Siete Cerros and Chinese Spring) with no frost tolerance (Table 3). Practically the same tendency could be observed for winter barley and with different degrees of frost tolerance and for spring barley with no frost tolerance (Table 4). A close positive correlation was found to exist between the frost tolerance of the given wheat and barley varieties, characterised by the LT_{50} values, and the quantity of 1.4 MD rRNA precursor.

The increase in the quantity of 1.4 MD rRNA precursor points primarily to an inhibition in the maturation processes of the ribosomes, especially in the final step, which is a very temperature-dependent one in varieties which have no or poor frost tolerance. In non-frost-hardy varieties the increase in the rRNA precursor level indicates that the disturbances caused by low temperatures do not act primarily through the $^{32}P_i$ uptake of the seedlings but rather by inhibiting the maturation processes of the ribosomes. The simplest interpretation of the results is to assume that frost-hardy wheat and barley varieties are generally characterised by a lack of disturbance of rRNA synthesis and of the maturation processes of the ribosomes at low temperature. This undisturbed processing is manifested as greater synthesis of the heavy and light stable cytoplasmic rRNAs (1.3 and 0.7 × 10^6 dalton). The relatively harmonious nature of this metabolism is destroyed as the frost hardiness of the varieties decreases and this then reaches expression in the higher quantity of 1.4 and 0.9 MD rRNA precursors synthesised.

The results obtained in the present experiments confirm observations made earlier in studies on correlations between low temperature and frost tolerance in terms of photosynthesis (Janda et al., 1996), polyamine synthesis (Rácz et al., 1996) and protein synthesis (Lasztity et al., 1994) in wheat.

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INFLUENCE OF WATER AND AIR TEMPERATURE ON COLD HARDINESS OF WHEAT

J. ABE and M. YOSHIDA

HOKKAIDO NATIONAL AGRICULTURAL EXPERIMENT STATION
SAPPORO, HITSUJIGAOKA, 062 JAPAN

The influence of water and air temperature on cold hardiness was examined in field-grown wheat, using freezing-tolerant (Valuevskaya), snow mould tolerant (P.I. 173438) and intermediate (Chihoku-komugi) varieties. A negative correlation between water content and freezing tolerance was not observed in all wheat varieties, as a comparatively low-temperature-sensitive snow mould tolerant variety had the lowest water content. However, the daily minimum air temperature regulated the water content and the cold hardiness. The water content in wheat plants decreased linearly with an increase in freezing tolerance under chilled conditions at temperatures above 0°C and then remained constant at subzero temperatures. The physical state of the crown water also changed from free water (weakly bound water) at chilling temperature (the first hardening stage) to bound water (tightly bound water) at subzero temperatures (the second hardening stage). Thus, the hardening process consists of two stages from the viewpoints of water and temperature, in agreement with Russian workers. As to the cell constituents associated with binding water molecules, the sugar profile in the crown changed correspondingly. Both simple sugars (mono- and disaccharide) and fructan (polysaccharide) increased during the first stage. In the second stage, the simple sugar content increased in both types of wheat, while the fructan content increased only in the snow mould tolerant variety.

Key words: winter wheat, cold hardiness, snow mould resistance, water content, sugar content

Introduction

Tissue water in herbaceous plants is involved not only in the development of cold hardiness but also in freezing injury. In general, there exists an inverse relation between water content and hardiness. Moriyama et al. (1995), however, reported that the freezing tolerance of herbage crops continued to increase without any corresponding reduction in water content in the later stage of the hardening process. This process has been proposed by Russian workers (Trunova, 1965) to consist of at least two stages, the first stage involving cold acclimation in the light at positive temperatures and the second stage being frost hardening. This may imply the importance of the physical state of water in relation to the hardening stage (Yoshida et al., 1997). Recent progress in NMR techniques allows us to analyse the mobility of water molecules in each population, as tissue water consists of several populations with different physical properties.

Sugars, whose accumulation during hardening is an essential component of the hardening process and whose content is commonly proportional to freezing tolerance, have also attracted attention in relation to changes in the physical state of water. Each soluble component, rather than the total sugar content, may affect the development of hardiness. Simple sugars (mono- and disaccharides), which are distributed throughout the cell even near to the membrane, are considered to be more effective for tolerance to freeze-induced dehydration than fructan (polysaccharide), most of which is localized in the vacuole. In contrast, freeze inhibitor polysaccarides have been predicted by Olien et al. (1986).

We have attempted to determine the hardening stage under natural conditions through changes in water and sugar content using wheat varieties showing different responses to winter stress (Abe et al., 1997).

Materials and methods

The seeds of three winter wheat (*Triticum aestivum* L.) varieties, Valuevskaya (highly freezing-tolerant), P.I. 173438 (snow mould-resistant) and Chihoku-komugi (moderately tolerant to winter stress), were sown in an experimental field at Sapporo (43°N lat.) during the third week of September from 1990 to 1995. Plants were sampled twice a month from October every year.

Freezing tolerance, expressed as LT_{50} (the temperature at which 50% of plants are killed), was determined using 50 crowns per variety (Moriyama et al., 1995). The water content of crown tissue measuring 2 cm in length was determined after drying at 80°C for 48h using 10 plants per variety, and expressed as g H_2O/g dry weight.

The vertical relation times (T_1) of protons in the water in intact crown tissue were determined at 23°C using a pulsed ^{1}H-NMR spectrometer (Yoshida et al., 1997). T_1 was determined using a $180°–\tau– 90°$ pulse sequence (τ = interval time between pulses) and calculated using spectra in the range of $0.01 \text{ s} \leq \tau \leq 0.1 \text{ s}$.

Sugar assay was carried out in 1993 (Yoshida, unpublished). The samples were preserved in a freezer at –80°C prior to sugar analysis. The soluble carbohydrates in crown tissues measuring 2 cm in length were extracted by boiling for 1.5 h. The solution was subjected to HPLC analysis using a Shodex KS 802 column warmed to 50°C. Refraction intensity detection peaks identified as fructose, glucose, sucrose and polysaccharide, that included isomers with more than DP 2, were calculated using a propylene peak as standard.

Results and discussion

Influence of air temperature on cold hardiness and water content

Illustrations are given of the changes in hardiness (Figs 1 and 3) and water content (Fig. 4) of Valuevskaya and Chihoku-komugi, since those of P.I. 173438 showed a similar tendency. Hardening commenced in early October, when daily minimum temperatures were below 10°C. Initially the LT_{50} values were around –5°C. Thereafter, hardiness increased almost linearly to reach a maximum hardiness in November to December. There was a great year to year variation in the maximum hardiness attained and the hardiness in 1990 and 1991 was distinctly inferior to that in the other years in both varieties. A comparison was therefore made of the daily minimum air temperatures of the five days preceding sample collection in two contrasting years (Fig. 2).

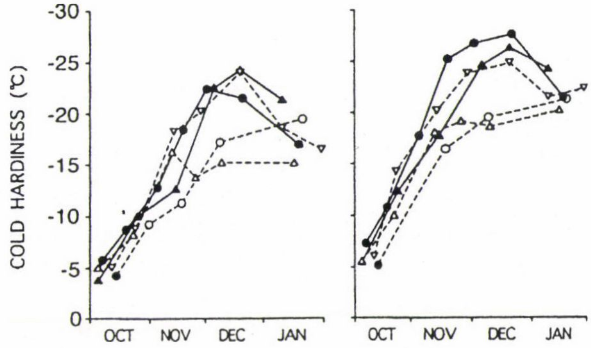

Fig. 1. Year to year variation in the development of cold hardiness (LT_{50}) in Chihoku-komugi (left) and Valuevskaya (right) in five years: 1990 (Δ), 1991 (O), 1992 (●), 1993 (▲), 1994 (∇)

The hardening induction time at 10°C was delayed for a week in 1990. In addition to a higher air temperature throughout the initial stage the fact that there were few days with freezing temperatures in the later stage caused much lower hardiness in 1990 than in 1992.

Further examinations were made to determine the influence of ambient temperature on hardiness for four consecutive years (Fig. 3). It is clear that each variety reacted in a predictable manner, with cold hardiness increasing linearly with decreasing air temperature, including freezing temperatures. The present data obtained from the field experiments support the findings of Trunova (1965) who carried out experiments at constant temperature.

In contrast to the development of hardiness, tissue water content decreased linearly with decreasing air temperature above 0°C and remained

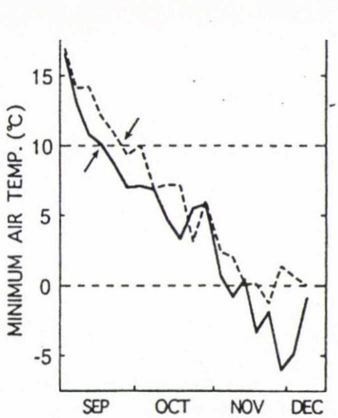

Fig. 2. Daily minimum air temperature during the hardening process in two contrasting years: 1990 (---), 1992(—). Arrows indicate the time of hardening induction

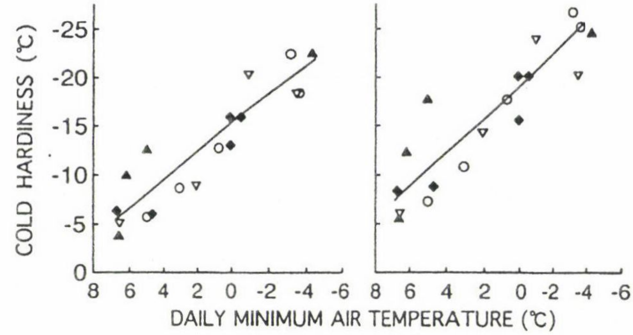

Fig. 3. Influence of daily minimum air temperature of the five days preceding sampling on the development of hardiness in Valuevskaya in four years: 1992 (○), 1993 (▲), 1994 (▽), 1995 (◆)

constant around 3g per g dry tissue below 0°C (Fig. 4). The balance between the water absorbed by the roots and that consumed in the form of evaporation and photosynthesis appears to be lost during the initial stage of hardening, resulting in decreasing tissue water content (Limin and Fowler, 1985). The fact that water movement is limited, together with little dry matter accumulation in the cell, may lead to constant water content in subzero temperature environments.

In accordance with Russian workers (Trunova, 1965), the existence of two hardening stages was concluded from the relation between water content and cold hardiness in field-grown wheat (Fig. 5). The first stage features an increase in hardiness accompanied by water reduction. Hardiness increased to a maximum level with little reduction in water content in the second stage.

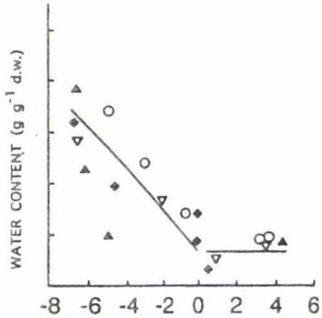

Fig. 4. Influence of daily minimum air temperature of the five days preceding sampling on water reduction in Valuevskaya in four years: 1992 (●), 1993 (▲), 1994 (▽), 1995 (◆)

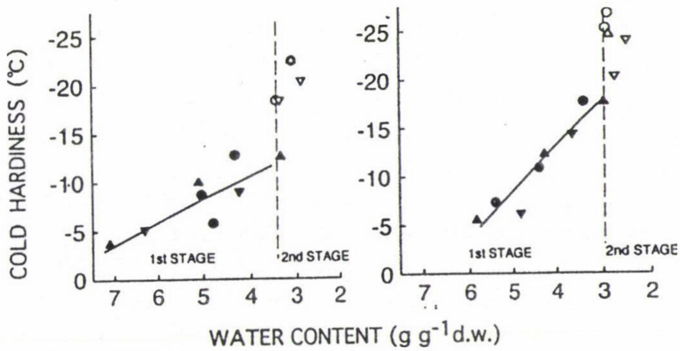

Fig. 5. Relationship between water content and cold hardiness in Chihoku-komugi (left) and Valuevskaya (right) in three years: 1992 (●○), 1993 (▲△), 1994 (▼▽). Closed and open symbols indicate data obtained above and below 0°C, respectively

The general concept of the negative correlation between cold hardiness and water content is applicable exclusively to the first stage of hardening. This implies the importance of the physical state of the water, together with the amount of tissue water.

Physical state of water in relation to hardening

Wheat remained in the tender state with an LT_{50} of about –5°C in the induction period of cold acclimation, but varietal differences in relaxation times (T_1) was observed (Fig. 6). An increase in cold hardiness was accompanied by a decrease in relaxation time throughout the first stage, mainly

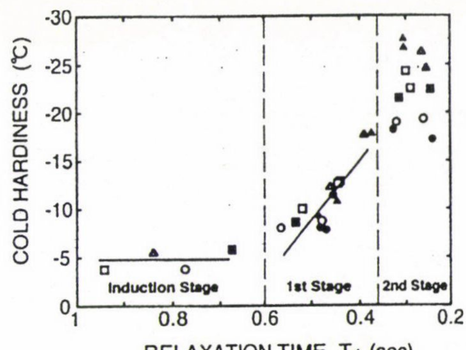

Fig. 6. Relationship between relaxation time (T_1) and cold hardiness in Valuevskaya (▲△), P.I. 173438 (●○) and Chikoku-komugi (■□) in two years. Closed and open symbols indicate data obtained in 1992 and 1993, respectively

because free water was completely used and cell water was bound, with an increase in cell materials. Drastic changes in the physical state of water together with a decrease in water content caused the important increase in cold hardiness at the transition period from the first stage to the second. In the second stage of hardening the relaxation times decreased slightly and showed similar values between the varieties despite a great varietal difference in hardiness. This suggests that tolerance to freeze-induced dehydration is of great importance rather than the ability to retain water, i.e. the avoidance of dehydration strain, in subzero temperature environments. The amount of unfreezable or "bound" water cannot explain the difference in hardiness between hardy and tender wheat varieties (Gusta et al., 1979). These findings encourage an examination of the mechanism which protects cells from freeze-induced dehydration from the viewpoint of cell substances.

Changes in sugar profile during hardening

Simple sugars and fructan contents increased with time in the first hardening stage (Fig. 7). After mid-November, when subzero temperatures prevailed, freezing-tolerant (Valuevskaya) and intermediate (Chihoku-komugi) varieties showed no increase in fructan, but only in simple sugars. On the other hand, the snow mould-resistant variety (P.I. 173438) showed a continuous increase in fructan, and consequently contained twice as much fructan as the other varieties in December, while the order of simple sugar content was consistent with the relative hardiness of the three varieties at this date. This change in the sugar profile in the second stage may play an important part in expressing tolerance to both low temperature (Trunova, 1965) and snow stress (Nakajime and Abe, 1994). In the latter case attention

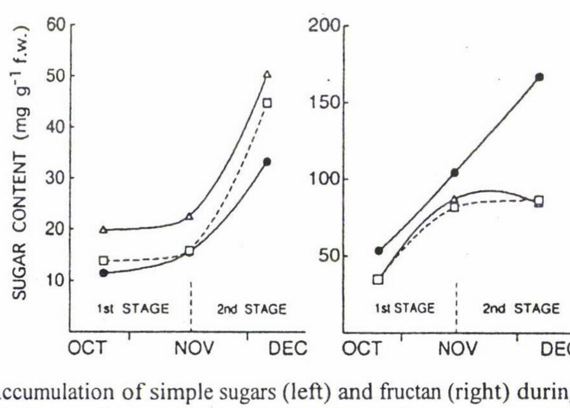

Fig. 7. Accumulation of simple sugars (left) and fructan (right) during hardening in Valuevskaya (Δ), P.I. 173438 (●) and Chikoku-komugi (□)

should be paid to P.I.173438, whose sugar metabolism is opposed to the general concept that a shift from fructan to simple sugars occurs in winter cereals after exposure to freezing temperatures (Livingston, 1996).

In conclusion, it was shown that plants having low water and high sugar content can express high freezing tolerance or high resistance to snow mould to a maximum level after exposure to freezing temperatures. These findings will be useful in determining the mechanism(s) involved in freezing tolerance and snow mould resistance.

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GENETIC AND ENVIRONMENTAL CONTROL OF WINTER SURVIVAL OF WINTER CEREALS

L. V. GUSTA, R. WILEN, P. FU, A. J. ROBERTSON and G. H. WU

CROP DEVELOPMENT CENTRE, UNIVERSITY OF SASKATCHEWAN
SASKATOON SK , S7N 5A8, CANADA

The winter rye (*Secale cereale*) cultivar Puma initiates freezing tolerance at warmer temperatures and attains a higher level of freezing tolerance than winter wheat (*Triticum aestivum*) or winter barley (*Hordeum vulgare*). Spring cereals, in contrast to the winter cereals, will not acclimate readily unless exposed to 2°C or less and only attain a maximum freezing tolerance of –8 to –9°C. Fully cold hardened winter cereals stored at cool non-sub-zero temperatures gradually lose freezing tolerance due to metabolic disorders (Olien, 1967). In contrast, the same seedlings can be stored at –3 to –4°C for up to six months with no measurable loss in freezing tolerance; however, a gradual loss in freezing tolerance occurs if the seedlings are stored at temperatures of –8°C or lower. The loss in freezing tolerance is correlated to the winter survival of the cultivar. These results may explain the mid-winter loss in freezing tolerance of cereals exposed to low non-lethal temperatures. Also, these results clarify why there is a low correlation between the minimum survival temperature used in controlled freeze tests and the winter survival of winter-hardy genotypes. Our results support the theory that winter kill in cereals is more a function of duration of exposure to sub-lethal temperatures rather than exposure to a minimum low temperature for a short duration. Gene expression studies are being correlated with freezing tolerance to identify markers for use in breeding programmes for the improvement of winter survival. Genes putatively associated with freezing tolerance (e.g. dehydrins, wcs 120) are expressed in both winter and spring cereal genotypes. However, the cold associated genes are only weakly and transiently expressed in spring genotypes. Therefore, the elucidation as to how the cold associated genes are regulated is essential for the improvement of winter survival. It has long been recognized that vernalization is closely linked to freezing tolerance. Spring-collected winter cereals, whose vernalization requirement has been met, cannot be hardened to their autumn level of freezing tolerance. In these seedlings, cold-associated genes (e.g. dehydrins) are only weakly or transiently expressed. Winter cereals collected in late autumn, at their maximum level of freezing tolerance, only reharden to the level of spring cereals following exposure to dehardening conditions. No correlation was observed between the level of freezing tolerance and the length of the vernalization period.

Key words: winter cereals, wheat, barley, rye, winter survival, cold-associated genes

Introduction

In western Canada, approximately 10 to 11 million hectares of spring wheat are grown each year, in contrast to only 100,000 hectares of winter wheat. Inconsistent winter survival is the primary factor restricting the northward expansion of winter wheat (*Triticum aestivum* L.) in the Great Plains of North America. The four main factors that limit winter cereal production in this area are: lack of the genetic potential to survive long periods of intense cold-induced desiccation; poor autumn conditions for acclimation; poor cultural management

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practices; and the unpredictable timing and duration of extreme cold temperatures. In western Canada, the major environmental factor limiting the production of winter cereals is prolonged low temperature extremes during the months of January and February when the lowest soil temperatures are traditionally recorded.

Prolonged periods of low sub-zero temperatures near –15°C during mid-winter have been identified as the primary cause of winter kill in winter wheat seedlings in western Canada (Gusta et al., 1994). During this cold period, winter wheat seedlings rapidly lose their acquired freezing tolerance and are killed at much higher temperatures compared to early winter (Gusta et al., 1997). Although winter wheat genotypes may have a similar minimum level of freezing tolerance in early November, seedlings of less winter-hardy genotypes held at low non-lethal temperatures lose freezing tolerance quicker than the hardy winter genotypes. The loss of freezing tolerance during winter poses two questions: first, what are the factors responsible for the loss of freezing tolerance and second, how does the plant protect itself from freeze-induced desiccation.

Completion of vernalization has been suggested to be a factor for the overwintering decline in freezing tolerance (Andrews, 1960; Roberts, 1990). Olien (1969) suggested that the primary cause for the decline in freezing tolerance at low above-zero temperatures is similar to the chilling injury experienced by warm season crops. Gusta and Fowler (1976) demonstrated that seedlings of Norstar winter wheat showed little or no loss in freezing tolerance following storage at –2.5°C for six months. However, seedlings maintained at lower sub-zero temperatures rapidly declined in freezing tolerance after 60 days of storage (Gusta et al., 1997). The lower the sub-zero temperature the faster the decline in freezing tolerance.

Recent evidence suggests that the low temperature-induced proteins are members of the LEA family (Robertson et al., 1994a,b; Fu, 1995). Dehydrins, members of the LEA family have been studied extensively and are highly correlated to the development of freezing tolerance. These proteins are highly hydrophilic and are thought to protect proteins, membranes and organelles from freeze-induced desiccation. The expression of a family of dehydrin genes was strongly correlated with the increased freezing tolerance in both spring and winter genotypes. The expression of these genes was detected sooner in the winter types, and dehydrin in mRNA accumulated to higher levels in winter cereals. The presence of dehydrin transcripts could be detected throughout the acclimation period in the winter cereals, but was only transiently expressed in spring cereals. In spring-collected field-grown winter cereals, a lower level of dehydrin mRNA was detected compared to the levels in late autumn. The level of dehydrin mRNA rapidly disappeared in seedlings grown in a glasshouse maintained at 23°C.

A third vital question concerning cold acclimation is how winter cereal seedlings perceive environmental cues to induce the hardening process. The signal-transduction pathway still remains a mystery. Low temperature-inducible genes are thought to be controlled by three separate signal pathways regulated by either low temperatures, ABA or a slight water stress (Gilmour and Thomashow, 1991, Nordin et al., 1991). Proof of these three pathways was based on the cold acclimation response of ABA-insensitive mutants, the use of ABA-deficient mutants, and the use of the ABA biosynthesis inhibitor, fluridone. The interaction of these three pathways is complicated, and unequivocal proof of three distinct pathways still awaits to be resolved.

While considerable attention has been given to cold-inducible cryoprotective proteins, photosynthetic studies conducted on cold-acclimating cereals have provided a different insight to the problem. The work of Huner and co-workers (Hurry and Huner, 1991) and Öquist et al. (1993) have eloquently shown that winter wheat cultivars can be distinguished from spring cultivars by their ability to keep fixing carbon into sucrose and fructans at low acclimating temperatures. The differential capacity of winter versus spring cultivars to adjust CO_2 assimilation rates was associated with higher levels of sucrose-phosphate synthase activity (Savitch et al., 1997). This research re-vitalizes the work conducted by Olien (1967) on the cryopreservative role of fructans which modify ice growth.

The objective of this study was to address the three questions discussed above and to obtain a better understanding of the role of vernalization in the maintenance of freeze tolerance and in the triggering of cold hardiness.

Materials and methods

Plots of winter cereals were seeded the first week of September with a hoe drill as described by Gusta et al. (1997).

A polyclonal dehydrin antibody (courtesy of Dr. T. Close, University of California – Riverside) was used for the detection of dehydrin-like proteins as described previously by Robertson et al. (1994a). Transcripts encoding dehydrin genes were detected by Northern analysis (Robertson et al., 1994b).

To determine the role of vernalization in controlling freezing tolerance plants were collected either in late autumn or early spring (late April). The freezing tolerance of the crown was determined at the time of sampling, and after 7, 10 and 14 days' storage at 25°C in a glasshouse. Rehardening of seedlings following this period of re-growth was performed in controlled environment chambers using the following schedule: 7 days at 7/5°C, 16 h photoperiod (PP); 7 days at 5/2°C, 14 h PP; 14 days at 2/0°C, 12 h PP and finally storage at –3°C for up to 21 days.

Seedlings grown hydroponically at the two- to three-leaf stage were partially cold acclimated for 14 d. The hardening protocol was 3 days at 7/5°C, 16 h PP; 3 days at 5/3°C, 14 h PP and 8 days at 2/0°C, 12 h PP. The seedlings were subjected to 25/23°C, 16 h PP for either 7, 10 or 14 days for dehardening. Following each period the seedlings were rehardened for 21 to 28 days at 2/0°C, 12 h PP.

To determine the degree of vernalization saturation, seedlings of both winter rye and wheat were collected from the field on November 1 and either transplanted into soil in a glasshouse maintained at 23°C ± 2°C with a 16 h PP or transferred to a controlled environment cabinet maintained at 7/5°C with a 12 h PP period. Seedlings were removed from this cabinet after 7, 14, 21 and 28 days and transferred to the glasshouse as described above. The number of days to flowering was recorded for each treatment and genotype.

Results and discussion

The effect of temperature on the maintenance of autumn-acquired freezing tolerance is depicted in Fig. 1. Seedlings held at –2.5°C show little or no loss in hardiness, whereas there is a gradual loss in freezing tolerance of seedlings held at either 4°C or –4°C. The most dramatic loss occurs in seedlings held at –15°C. An important finding in this study was that not all cultivars respond to sub-freezing temperatures the same way. Gusta et al. (1997) revealed there was little or no difference in the freezing tolerance of Norstar (a very hardy winter wheat) and Rose (a moderately hardy winter wheat) in early December. However, seedlings of the winter cultivar Rose held at –8°C died after 60 days, whereas seedlings of Norstar were still alive after 90 days. Thus, although seedlings of different genotypes may have a similar LT_{50} when frozen at 2°C h^{-1}, they may differ dramatically in their ability to tolerate long periods of sub-lethal temperatures. The results suggest the primary cause of winter kill in western Canada is the result of freeze-induced desiccation.

Several researchers have suggested that vernalization is involved in the overwinter decline in freezing tolerance (Andrews, 1960; Roberts, 1990). Four cultivars of winter wheat were evaluated to determine the relationship between freezing tolerance, the maintenance of freezing tolerance and time to vernalization saturation. Norstar and Mesa have a short time to vernalization saturation but differ dramatically in winter survival. In late autumn, both cultivars can tolerate –23°C; however, seedlings of Norstar and Mesa stored at –4°C for approximately 130 days tolerated –9 and –5°C, respectively. The cultivars Aura and Jokkienen have a long vernalization saturation time (at least two weeks longer than Norstar) but have a higher LT_{50} than Norstar following storage at –4°C. The winter rye cultivar, Puma, has the shortest time for vernalization saturation and is the most cold-hardy winter cereal. The results suggest that the length of time to vernalization is not correlated with either the degree of freezing tolerance attained or the length of time that freezing tolerance is maintained. The loss of freezing tolerance over winter is primarily a function of the intensity of the sub-zero temperature.

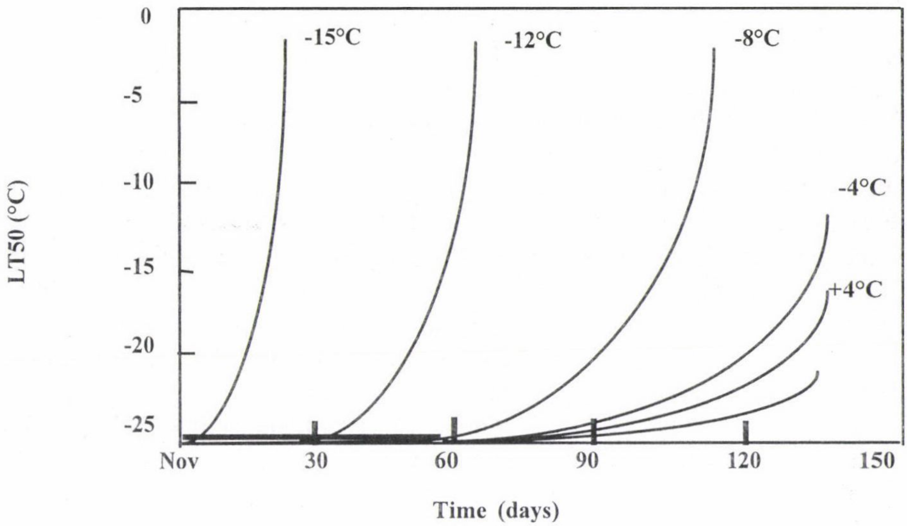

Fig. 1. The effect of temperature on the freezing tolerance of fully hardened Norstar winter wheat seedlings

Low temperatures and short photoperiods are the general environmental cues that either induce or upregulate the genes involved in cold acclimation; however, the conversion of these stimuli into a response is not understood. Seedlings of winter cereals growing in the field in early summer can tolerate –5 to –9°C, even though they are not exposed to cold acclimating conditions (Table 1). Seedlings of winter cereals grown in pots subjected to a mild water stress (–15 to –20 bars) can tolerate –7 to –9°C. A root drench of 100 mM ABA to seedlings of Puma rye grown at 20°C will increase the freezing tolerance of the crowns 6 to 8°C. Seedlings grown under non-stressful conditions in a controlled environment cabinet only tolerate –2 to –5°C. Therefore, stresses other than low temperature in the field (e.g. wind, water stress) induce a level of freezing tolerance comparable to spring cereals grown under cold acclimating conditions.

Table 1

Effect of environment and ABA on the cold hardiness of Puma rye crowns

Treatment	LT_{50} (°C)
25/20°C Glasshouse	–3
25/20°C Water stress	–10
25/20°C, 100 μM ABA	–8
Field (July)	–9
2/0°C Growth chamber	–28
Field (November)	–33

Freezing tolerance is an inducible character in winter cereals with low temperature acting as the primary factor of initiating the process (Fig. 2). However, the signal transduction pathway for the induction of the cold-regulated genes is unknown. It is highly possible that secondary signals such as ABA, sucrose, fatty acids and water, which are produced in response to the primary signal (cold), are involved. All of these factors are known to regulate gene expression. Fu (1995) observed a decrease in water potential in seedlings of winter cereals but not in seedlings of spring cereals exposed to cold hardening conditions. A transient increase in ABA was observed in potatoes exposed to low temperatures (Chen et al., 1983). Low temperature itself, or secondary factors, can result in membrane perturbations or a conformational change in either membranes and/or proteins and/or an ABA hormone receptor. These events can result in the activation or release of transacting factors or the production of transcriptional factors that upregulate genes involved in cold acclimation. Release of a second messenger such as calcium or jasmonic acid can result in a cascade effect that activates certain enzymes, e.g. activation of kinases or phosphates that could result in the release of a suppressor that down-regulates the cold hardiness genes.

Fu (1995) demonstrated that Puma rye initiated cold acclimation at warmer temperatures than either winter wheat or winter barley. Thus, the very cold-hardy winter cereals such as Puma rye have a longer period for cold acclimation than the less hardy winter cereals. Spring cereals of wheat, rye and barley did not increase in freezing tolerance until the temperature was lowered to 2°C or colder. These results suggest that the trigger for inducing the promoters controlling the cold-regulated genes in winter rye is much more sensitive than in the other cereals. In the future it is expected that elucidating the initiation of cold acclimation will be an active area of research.

A strong association has been established between the degree of vernalization and the degree of freezing tolerance that can be achieved in winter cereal seedlings (Andrews, 1960; Roberts, 1990). Wilen et al. (1997) reported that seedlings of Norstar winter wheat and Puma winter rye collected in early spring only hardened to the level of spring cereals upon exposure to cold acclimating conditions. The pattern of dehydrin accumulation in these seedlings was similar to the levels obtained during the cold acclimation of spring cereals. Although there was a decrease in crown water content and an increase in simple sugars there was no decrease in the water potential in seedlings of winter cereals exposed to cold hardening conditions in the spring The level of freezing tolerance attained was equivalent to that of spring cereals (LT_{50} –7 to –9°C).

Field acclimated seedlings in early November were tested for their ability to reharden following a period of dehardening for 7 and 14 days (Table 2). In contrast to the spring collection, seedlings of Norstar winter wheat and Puma winter rye dehardened for 7 days still retained their ability to reharden, though not to the same level as prior to dehardening. Seedlings held at 23°C for 14 days

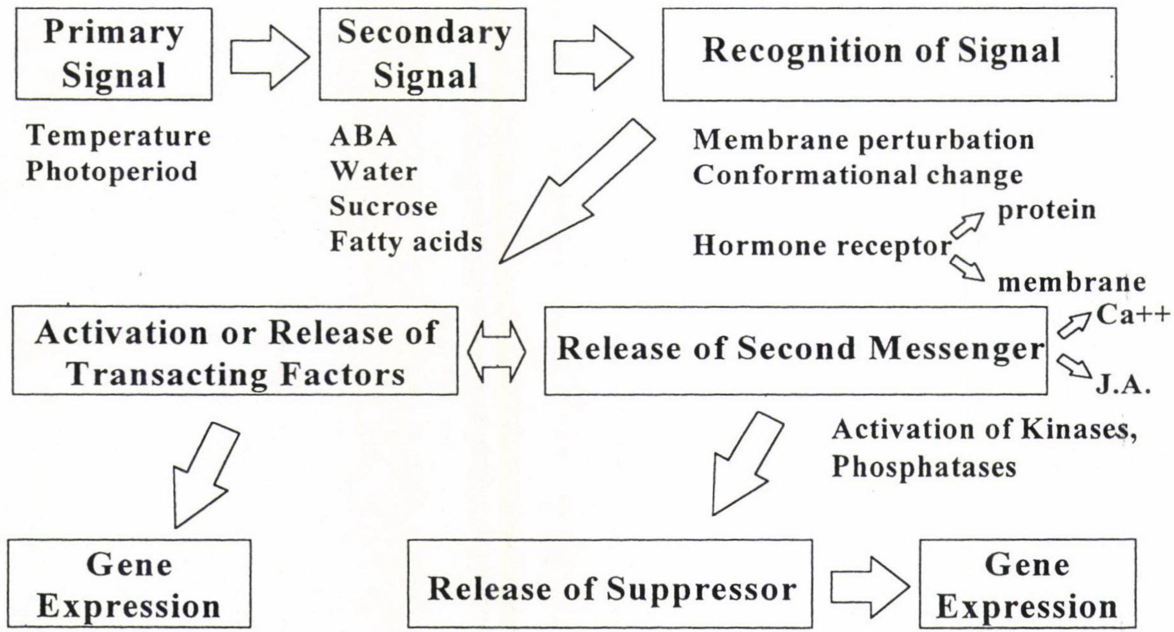

Fig. 2. Proposed signal-transduction pathway for the induction of freezing tolerance

Table 2
Dehardening and rehardening of Norstar winter wheat and Puma winter rye collected in late autumn

Cultivar	Autumn	LT$_{50}$ (°C)			
		7d, 23°C	7d, 5/2°C	7d, 2/0°C	6 wks, –4°C
Norstar	–23	–8	–9	–14	–15
Puma	–28	–10	–21	–20	–20
		14d, 23°C			
Norstar	–23	–2	–5	–10	–10
Puma	–28	–2	–13	–17	–18

did not reharden to the same level as seedlings only dehardened for 7 days at 23°C. Seedlings of Norstar were less responsive than Puma seedlings.

Seedlings of winter wheats varying in winter hardiness and two hardy cultivars of winter rye were transplanted from the field in early November to a glasshouse maintained at 23°C. Additional seedlings were vernalized for up to four weeks at 6/3°C, 12 hour photoperiod (Table 3). The date of flowering was recorded for each cultivar. Briefly the most cold-hardy genotypes, Puma winter rye and Norstar winter wheat had the shortest time to flower. An additional week of vernalization treatment only reduced the time to flower by 4 to 6 days. Thus vernalization is saturated prior to the onset of winter for these very hardy genotypes. The moderately winter-hardy winter wheats, Aura and Jokkienen flowered 55 to 70 days later than the cultivars Norstar and Puma. An additional two weeks of vernalization conditions were required to saturate the vernalization requirement. As demonstrated earlier there was no correlation between the freezing tolerance of the seedlings and the length of time required for vernalization saturation. This is particularly exemplified by Norstar and Puma seedlings which have a very short period to meet the vernalization saturation time but are the hardiest winter wheat and winter rye cultivars, respectively. The cultivar Jokkienen requires a very long period at low temperatures to meet the saturation requirement for vernalization, however, it is a relatively non-hardy winter wheat.

The cultivars Katepwa spring wheat, Norstar, Aura and Jokkienen winter wheats and Puma winter rye were cold hardened for only 14 days and then tested for their ability to reharden following 7, 10 and 14 days of dehardening. In contrast to fully hardened seedlings collected in late autumn, partially hardened winter wheats did not harden to their full potential. The longer the seedlings were held at non-hardening temperatures the less freezing tolerance could be established. Seedlings of Puma rye rehardened to –20°C following a period of 7 days of dehardening, however, seedlings dehardened for 14 days could only tolerate –11°C after a period of rehardening at 2/0°C for 3 weeks. Surprisingly Katepwa spring wheat, which does not have a vernalization requirement, did not reharden following dehardening for 7 days.

Table 3
Days to flower of winter cereals collected from the field on Nov. 1

Cultivar	LT_{50} (°C)	Days to flower at 23°C 6/3°C (12 h photoperiod)[1] (days)				
		0	7	14	21	28
Rye						
Puma	−30	37	31	32	27	32
Rifle	−30	41	34	34	34	34
Wheat						
Norstar	−24	49	53	45	48	44
Mesa	−23	41	41	34	41	39
Jokkienen	−17	115	74	74	59	60
Aura	−18	97	81	62	59	61

[1]Seedlings collected from the field on Nov. 1 received an additional vernalization treatment for up to 28 days

Summary

1) The loss of freezing tolerance in seedlings of winter cereals is regulated by the intensity of the sub-zero soil temperatures.
2) Vernalization does not appear to play a major role in the level of freezing tolerance attained and the maintenance of freezing tolerance in seedlings of winter cereals.
3) Vernalization does not appear to play a major role in the rehardening of seedlings of winter cereals.
4) Seedlings of Puma winter rye initiate cold hardening at a warmer temperature than either seedlings of winter wheat and winter barley or spring cereals. Therefore, the trigger for cold acclimation in winter rye is very sensitive to environmental conditions.

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DISSECTING THE COMPONENTS OF WINTER HARDINESS IN BARLEY

P. M. HAYES[1], T. H. H. CHEN[1], W. POWELL[2], W. THOMAS[2], Z. BEDŐ[3], I. KARSAI[3] and K. MÉSZÁROS[3]

[1]DEPARTMENT OF CROP AND SOIL SCIENCE AND DEPARTMENT OF HORTICULTURE
OREGON STATE UNIVERSITY, CORVALLIS, OR 97331, USA
[2]SCOTTISH CROP RESEARCH INSTITUTE, DUNDEE, DD25DA, SCOTLAND
[3]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, P.O. BOX 19, HUNGARY

Genomics offers new approaches to understanding the genetic control of complex phenotypes, such as the components of winter hardiness in cereals. Genetic determinants of these components can be effectively manipulated if their linkage and pleiotropic relationships with determinants of other economically important phenotypes are known. Information on the genome location of loci determining agronomic, winter hardiness and malting quality phenotypes were integrated from eleven barley mapping populations. Map coordinates of these loci will be of assistance in developing varieties with unique attributes and will serve as a basis for cloning. The presence of coincident and linked QTL indicates that genes determining winter hardiness-related traits must be viewed in the context of the complete phenotype.

Key words: barley, winter hardiness, genomics, linkage mapping, QTL

Introduction

Winter hardiness in barley is determined by a number of characteristics, including low temperature tolerance, response to photoperiod and response to vernalization. Genomics offers new approaches to understanding the genetic control of these complex phenotypes. For example, eight years ago it was still a point of discussion that vernalization and low temperature tolerance were correlated, but not inseparable, characteristics (Doll et al., 1989). The basis of this relationship is now understood to be due to linkage on barley chromosome 7 (Mészáros and Hayes, 1997). This linkage relationship was confirmed on the homoeologous group 5A chromosome of wheat (Galiba et al., 1995). To date, genomic analyses in barley have been based on genetic maps and their use in detecting quantitative trait loci (QTL). In another eight years, we will see new horizons in winter hardiness genetics as progress is made in comparative QTL mapping, physical mapping and gene characterization.

Materials and methods

Map positions of loci determining components of winter hardiness, grain yield and malting quality were obtained from published reports on eleven mapping populations. The numbers after each of the following sources used in generating the summary refer to Fig. 1 [Backes et al., 1995 (8); Hayes et al., 1993 (10); Hayes et al., 1996a (2); Hayes et al., 1996b (5); Karakousis, 1996

(3,4,6); Kjaer et al., 1995 (11); Laurie et al., 1995 (12); Oziel et al., 1996 (5); Tinker et al., 1996 (7); Thomas et al., 1996 (1)]. The QTL were positioned on a molecular marker linkage map developed in the Harrington × Morex population (Hayes et al., 1997). The integrated barley map of Qi et al. (1996) was used to identify common flanking markers for the various populations. QTL positions can only be considered approximate. The reader is urged to consult original reports for more detailed information on QTL position, allele phase and magnitude of effect. QTL were positioned on the skeleton map only when QTL for the same trait were detected in two or more populations or when QTL for two or more different traits were reported at that position.

Results and discussion

The complexity of the genetic control of the components of winter hardiness, and the integral relationships of these loci with determinants of agronomic and quality phenotypes, is apparent in the cartoons of chromosomes 1, 2, 5 and 7 shown in Fig. 1. These chromosomes were chosen for illustrative purposes. Similar patterns were observed on chromosomes 3, 4 and 6. Coincident QTL can be due to either linkage or pleiotropy. There is clearly a relationship between the number of loci determining each trait and the challenge that trait poses in terms of phenotyping. More loci are reported to determine response to photoperiod than low temperature tolerance, although the latter is a more formidable character to phenotype than the former. The implication is that the quality of the phenotype data may be a larger determinant of the magnitude of the heritability estimate than the number of genes involved. Controlled environment facilities – such as the Martonvásár Phytotron – can be invaluable in maximizing heritability and consequently the sensitivity of the QTL analysis.

The QTL information summarized in Fig. 1 has immediate practical implications and lays the groundwork for future investigations. Most of the genetic variation for low temperature tolerance in genetic stocks derived from the matings of low temperature tolerant × low temperature susceptible genotypes is due to allelic variation at a single locus or complex locus (Mészáros and Hayes, 1997; Galiba et al., 1995). This would account for the lack of selection response for low temperature tolerance and indicates that major breakthroughs in low temperature tolerance are not likely in matings of adapted germplasm. However, comparative QTL analyses, such as those underway at the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, will be useful in extending our knowledge of winter hardiness genetics to winter barley germplasm and may reveal loci that can be pyramided to achieve additive improvement in low temperature tolerance. Information regarding the genetic structure of a range of winter germplasm will allow for the efficient development of genotypes with unique combinations of vernalization, photoperiod and low temperature response. For example, one approach to minimizing the risk of winter barley production in temperate environments with intermittent winter injury would be to develop varieties with low temperature tolerance, sensitivity to short photoperiods, but lacking in vernalization requirement.

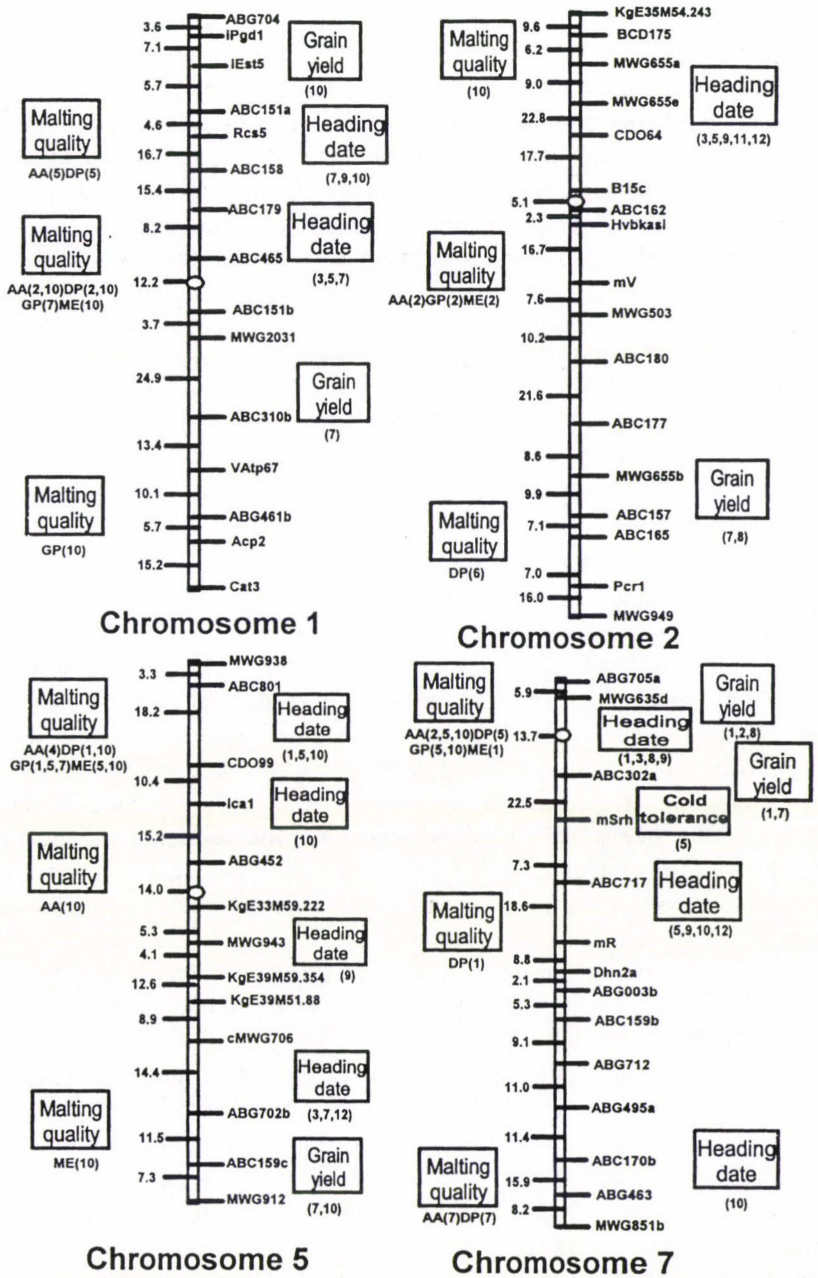

Fig. 1. Linkage maps of chromosomes 1,2,5 and 7 based on the Harrington × Morex population, showing QTL for components of winter hardiness, malting quality and grain yield reported in 11 barley mapping populations (see text for citation codes).
Malting quality: AA = alpha amylase; DP = diastatic power; GP = grain protein; ME = malt extract

In this way, should there be a significant winter kill event, the grower could re-seed with the same variety in the spring. At the same time, through marker-assisted selection, current levels of low temperature tolerance can be efficiently introgressed into spring genotypes with desirable quality and/or agronomic profiles. Barley varieties must also possess acceptable agronomic and quality profiles. Information regarding linkage and/or pleiotropic relationships will be invaluable in designing and implementing breeding strategies.

Genomic analyses of the components of winter hardiness will serve as a resource for positioning cloned candidate genes on linkage maps and relating them to QTL and Mendelian loci. For example, extensive efforts are underway to clone an array of dehydrin genes and relate their map position to winter hardiness QTL (van Zee et al., 1995; T. Close, personal communication). Likewise, genome coordinates can be used as a platform for map-based cloning. These endeavours will be challenging but they will begin to address fundamental issues in genome evolution and gene expression. The chromosome 7 region where low temperature, vernalization and photoperiod reaction QTL are reported has one of the more tractable physical: genetic distance ratios (less than 2 Mbp/cM) (Kunzel and Korzun, 1996).

More generally, the evolution of the components of winter hardiness is an intriguing subject. Within the *Triticeae*, there are homoeologous relationships and a range of low temperature responses that are not linearly related to ploidy level. Throughout the world, winter wheats are grown in regions where winter barley production is not economical, and yet accessions of rye are superior to hexaploid wheat. Syntenic relationships among the *Gramineae* have been established, allowing for the alignment of portions of barley chromosome 7 with rice chromosomes 3, 9 and 12 (Saghai-Maroof et al., 1996). Photoperiod response is common to the three species, yet vernalization response and extreme low temperature tolerance are not found in maize or rice. Exploration of these genomes at the microsynteny level should lead to an understanding of the evolution of vernalization and low temperature tolerance in relation to photoperiodism.

A complete understanding of genome structure and expression may ultimately allow for the development of winter barley varieties that are more resistant to low temperature stresses. This will enhance agricultural diversity and sustainable, economic crop production. However, pleiotropy, linkage and epistasis will preclude simple solutions. The Martonvásár Phytotron will be an invaluable tool for systematically dissecting the physiology and genetics of adaptation, growth habit and low temperature tolerance in cereals.

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EFFECT OF ABIOTIC AND BIOTIC ENVIRONMENTAL FACTORS ON THE FROST RESISTANCE OF WINTER CEREALS

O. VEISZ

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, HUNGARY

Studies on the winter hardiness and frost resistance of winter cereals have been underway in the Martonvásár institute for several decades. This research covers two major fields: the clarification of the role of ecological factors in the development of frost resistance, and work on the genetic and physiological background of frost resistance. The present paper describes experiments designed to study the effect of an abiotic environmental factor, i.e. increased atmospheric CO_2 concentration, and that of a biotic factor, i.e. bunt infection, on the hardening and frost resistance of cereals, as well as a research result showing changes in the interaction between environment and genotype. The frost resistance studies carried out in the Martonvásár phytotron led to the following conclusions:

- An increase in the atmospheric CO_2 concentration had a beneficial effect not only on the initial development of cereals, but also on the process of cold hardening, so plants hardened at an atmospheric CO_2 concentration of 750 $\mu mol \cdot mol^{-1}$ had a higher survival rate after freezing than those in the control treatment at the normal level of 350 $\mu mol \cdot mol^{-1}$.
- A joint examination of biotic and abiotic stresses was carried out by analysing the relationship between bunt infection and frost resistance. The frost resistance of bunt-infected plants was lower than that of healthy plants. As the result of infection there was an increase in the killed plant rate, and a close positive correlation ($r = 0.890$) was found between the increased frost kill due to infection and the degree of susceptibility to bunt.
- The dominance relations were found to be dependent on the freezing temperature in the F_1 generation. While at –12°C frost resistance was dominant, frost sensitivity became dominant when the plants were frozen at –18°C.

Key words: cereals, frost resistance, hardening, atmospheric CO_2, bunt infection, freezing temperature

Introduction

The winter hardiness of plants depends on their genetically determined adaptability and on the abiotic and biotic environmental factors which influence the manifestation of their traits.

Among the environmental factors, temperature, light, precipitation, and the quantitative and chronological distribution of these factors have an influence on the development of frost resistance in the course of hardening (Lelley, 1956; Rajki, 1980). The temperature is of key importance in the hardening process (Gusta and Fowler, 1976; Roberts and Grant, 1968). According to Olien (1967) the optimum hardening temperature for winter cereals is around 3°C.

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Depending on the preliminary growth and hardening conditions, the results of freezing are greatly influenced by the duration of the cold effect, the frequency of freezing and thawing, and the moisture content of the plant tissues (Pomeroy et al., 1975).

Less is known on the role of atmospheric CO_2 concentration in the development of frost resistance in cereals. Due to increasing concentrations of carbon dioxide, methane, nitrogen oxides and other gases in the earth's atmosphere over the last decades an ever greater greenhouse effect has developed (White, 1992). Plant responses to higher CO_2 concentrations have been studied by several authors (Kimball, 1983; Mitchell et al., 1993; Thompson and Woodward, 1994). The majority of cereals respond positively to "CO_2 fertilisation". A doubling of the CO_2 level leads to a 10–15% increase in biomass production if other environmental conditions do not limit growth. As regards the effect of increased CO_2 concentrations on the frost resistance of plants, Dalen et al. (1996) found that the frost resistance of Norway spruce (*Picea abies*) samplings grown in a phytotron was significantly better at an atmospheric CO_2 concentration of 700 μmol mol^{-1} than at normal (400 μmol·mol^{-1}) concentration.

Bunt can be found in all regions of the world where wheat is grown and causes damage to a greater or lesser extent (Vánky, 1985; Jonsson, 1992; Gaudet et al., 1993). At the beginning of the century, considerable damage was recorded in Hungary (Hegyi, 1911), but obligatory seed dressing, which is usually carried out with great care, has led to the suppression of the pathogen, which has only been observed sporadically in wheat fields over the last few decades (Papp, 1991).

The damage caused by bunt (*Tilletia foetida*, Wallroth, Liro and *T. caries*, Tulasne), may be considerable and may take several forms (Hegyi, 1911; Podhradszky, 1962). One important aspect of the damage is that the frost sensitivity of the wheat may increase (Podhradszky, 1962; Babayants, 1988).

Changes in environmental factors (e.g. freezing temperature) have an effect on the manifestation of genetically determined traits. While some authors found freezing resistance to be the result of recessive genes with additive effects (Law and Jenkins, 1970), others reported both additive and non-additive effects, controlled by both recessive and dominant genes (Eunus et al., 1962; Rohde and Pulham, 1960). Amirshahi and Patterson (1956) found additive effects with no gene interaction.

Le et al. (1986) reported a difference in the ranking obtained for the survival of winter wheat cultivars depending on whether a high or low intensity freezing test was used. In a severe test, Rohde and Pulham (1960) reported that hardiness was controlled by recessive genes, while in a less severe test hardiness was dominant.

The present paper describes experiments designed to study the effect of an abiotic environmental factor, i.e. increased atmospheric CO_2 concentration, and that of a biotic factor, i.e. bunt infection on the hardening and frost resistance of cereals, as well as a research result showing changes in the interaction between environment and genotype.

Materials and methods

The experiments were carried out in the phytotron of the Agricultural Research Institute of the Hungarian Academy of Sciences. Winter wheat varieties originating from the major wheat-growing regions of the world, and Martonvásár-bred winter wheat, triticale and rye varieties with genetically different frost resistance values were used in the experiments.

For the phytotronic frost test, germinated wheat seeds were sown in wooden boxes measuring 42 × 30 × 13 cm. The growth medium was a 3:1:1 mixture of garden soil, humaszka (humus-containing additive) and sand. The plants were grown in nine rows per box, with 20 plants per row. Each box represented one replication and there were four replications in each experiment.

The preliminary growth stage lasted for six weeks, during which period the temperature was gradually reduced once a week, to simulate natural conditions. Preliminary growth was followed by two-phase hardening. The first phase took place in the autumn–winter chamber used for preliminary growth and the temperature fluctuated daily between +3°C and –3°C for a week. The second, 4-day hardening phase was carried out in the frost testing chamber at –4°C immediately prior to freezing.

The temperature was gradually reduced and freezing took place at –15°C for 24 hours. The temperature was then raised by 1°C an hour and after the two-day thawing period the boxes were transferred to growth benches. Further growth took place at a day/night temperature of 17/16°C with a 14-hour day and 125 mmol .mol^{-1} photon flux for three weeks. At the end of the third week plants which had survived freezing and started developing could be readily distinguished from those which had died. A detailed description of the M29 programme used in the frost testing method was reported by Tischner et al. (1997).

The above method was used as the basic programme and the ecological factors studied were changed as required by the experimental aims.

The varieties included in the experiment in which the effect of bunt were determined were infected with 0.5 g local pathogen population spore mixture per 1000 seeds. When the weather was favourable for bunt development, usually in late October or early November, 250 seeds per variety or line and per plot were sown in 3-row plots measuring 1 m in length, with an area of 0.5 m^2 in 3 replications. The degree of susceptibility was determined after harvesting as the percentage of infected ears (Szunics and Szunics, 1990). The frost resistance of healthy plants and of plants, infected with bunt in the same way as in the field experiment, was studied in the phytotron. Infected and healthy seeds were sown in separate wooden boxes.

The evaluation of the experimental data was carried out using two-factor analysis of variance. Differences between means were tested by least significant differences (LSD) (P=0.05, 0.01 and 0.001).

Results

Effect of atmospheric CO_2 concentration on the frost resistance of cereals

The effect of increasing atmospheric CO_2 concentration on the cold hardening of young plants was studied under phytotronic conditions. The experiments were carried out simultaneously in two plant growth chambers. The growth conditions, which simulated the average autumn weather conditions in Hungary, were identical in the two chambers, with the exception of the atmospheric CO_2 content. In one unit the normal CO_2 level (350 μmol mol^{-1}) was programmed, while in the other this level was doubled (700 μmol mol^{-1}).

With the exception of one variety an increase in the atmospheric CO_2 content had a favourable influence on the hardening process, which meant that more plants survived than under normal conditions (Table 1). In the course of preliminary growth trends in leaf area and dry mass were recorded, as these are indicative of biomass production. For all the varieties a rise in the CO_2 concentration increased the leaf area. The dry mass was also greater for most of the varieties compared to the control treatment.

Table 1
Effect of atmospheric CO_2 concentration on the initial development and frost resistance of cereals

Variety	Origin	Leaf area (cm^2)		Plants surviving freezing (%)	
		350	700	350	700
		CO_2 μmol mol^{-1}		CO_2 μmol mol^{-1}	
Winter wheats					
Fredrick	N. America I.	7.69	10.71***	42.5	58.7
Karl	N. America II.	5.05	5.67	81.2	92.5
Alba	N. Europe	5.25	6.53*	20.0	36.3
Mv 15	C. Europe	5.20	5.97	81.2	91.2
Libellula	S. Europe	7.80	8.06	3.7	2.5
Thesee	W. Europe	8.02	7.22	1.2	11.2
Bezostaya 1	E. Europe	7.11	7.22	80.0	80.0
Gerek	SE. Europe	6.57	6.29	3.7	31.2**
Winter triticale					
Presto		6.01	7.60*	38.7	63.7**
Winter rye					
Motto		9.95	9.74	93.7	94.1
Mean		6.86	7.50*	44.6	56.1*
$LSD_{5\%}$ between any two combinations		1.129		17.9	
$LSD_{5\%}$ between mean values		0.160		6.7	

*,**,*** significant at P = 0.05, 0.01 and 0.001, respectively

Effect of bunt infection on the frost resistance of wheat varieties

Table 2 presents data on the bunt resistance of 9 registered wheat varieties on the basis of a 3-year series of experiments. None of the varieties was resistant. Three were moderately resistant: 'Martonvásári 17', 'Fatima 2' and 'Martonvásári 23'. Two varieties were included in the moderately susceptible group (11–30% infection): 'Martonvásári 16' and 'Martonvásári 18'. A considerable proportion of the cultivated varieties were susceptible, being 30–50% infected ('Martonvásári 20', 'Martonvásári 19', 'Martonvásári 14') or very susceptible ('Martonvásári 21'). This can be attributed to the fact that no breeding against this pathogen is carried out in Hungary, which means that the presence of resistance or moderate resistance to bunt in a variety is due purely to chance (Szunics and Szunics, 1990).

Of the nine varieties tested, 'Martonvásári 16', 'Martonvásári 17', 'Martonvásári 18' and 'Martonvásári 20' have excellent frost resistance, that of 'Martonvásári 19', 'Martonvásári 21', 'Martonvásári 23' and 'Fatima 2' is good and that of 'Martonvásári 14' is medium (Table 2).

As the result of bunt infection, diseased plants of the varieties examined suffered to a greater extent from frost kill than healthy plants. Significantly more plants were destroyed in the case of seven varieties. In the majority of varieties, the greater frost kill as the result of infection was related to the degree of susceptibility. The only exception to this was 'Martonvásári 23', which has good resistance to bunt, yet suffered a significant reduction in the survival rate after freezing as the results of bunt infection. A close correlation (r = 0.890, significant at P = 0.05) was found between the increased frost kill due to infection and the degree of susceptibility to bunt.

Table 2

Effect of bunt infection on the frost resistance of wheat varieties, phytotron (–15°C)

Genotype	Infection (%) (average of 3 years)	Rate of plants killed (%)		
		Healthy plants	Infected plants	Difference
Martonvásári 14	39.8	37.6	63.2	25.6*
Martonvásári 16	29.1	8.9	29.0	20.1*
Martonvásári 17	5.0	7.8	25.3	17.5
Martonvásári 18	29.8	3.9	28.1	24.2*
Martonvásári 19	31.2	17.4	39.1	21.7*
Martonvásári 20	31.1	8.9	29.7	20.8*
Martonvásári 21	55.8	13.3	43.9	30.6***
Martonvásári 23	10.6	18.7	38.7	20.0*
Fatima 2	6.5	23.6	34.7	11.1
Mean		15.6	36.8*	21.2*
$LSD_{5\%}$	8.2	18.9	19.5	19.2

*,*** significant at P = 0.05 and 0.001, respectively

Effect of freezing temperature on the direction of dominance of frost resistance

The direction of dominance in the control of frost resistance was studied when winter wheat parental lines with different degrees of frost resistance and their F_1 hybrids were frozen at various freezing temperatures.

The results obtained at the four different freezing temperatures are presented in Figs 1 and 2. In the first combination, the variety Cheyenne was one of the most frost-resistant varieties, while GK Szeged was one of the poorest (Fig. 1). At all four freezing temperatures there was a significant difference between the varieties: while Cheyenne had a very low killed plant rate even when tested at −18°C, GK Szeged suffered considerable frost damage even at −14°C. The difference in frost resistance between the two parents increased as the freezing temperature was reduced. The frost resistance of the F_1 generation had a value between those of the two parents. At −12°C the percentage survival of the F_1 hybrid did not differ significantly from that of the better parent, while it was significantly higher than that of GK Szeged. The frost resistance of the F_1 generation was significantly lower than that of the better parent when frozen at a temperature of −14°C or below. The frost resistance of the F_1 population was considerably higher than that of the GK Szeged parent when freezing took place at −12, −14 or −16°C, while at −18°C the difference decreased and the deviation was no longer significant.

In the case of the other parental pair, Martonvásári 4 × Martonvásári 10, a similar tendency was observed (Fig. 2). In this combination Martonvásári 4 had excellent frost resistance, while that of Martonvásári 10 was moderate. Due to this, the difference between the two varieties was significant at −14, −16 and −18°C.

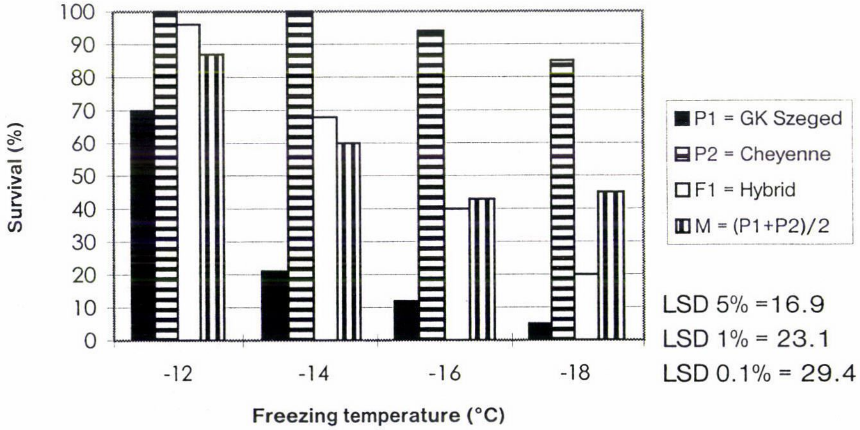

Fig. 1. Frost resistance of GK Szeged and Cheyenne parents and their F1 hybrids

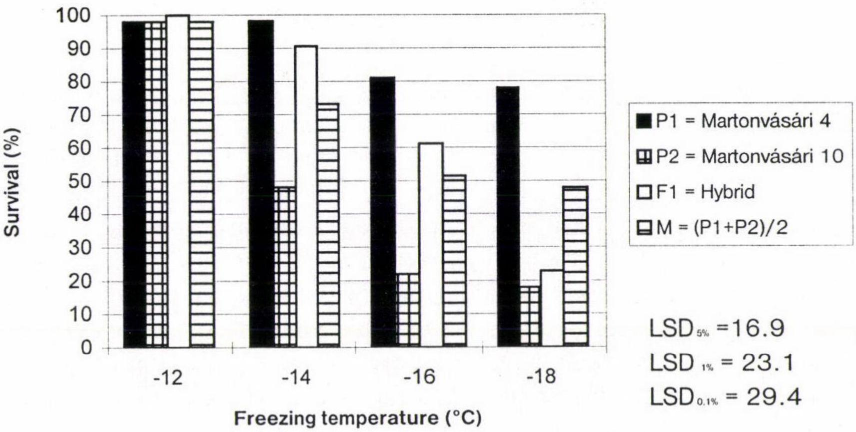

Fig. 2. Frost resistance of Martonvásári 4 and Martonvásári 10 parents and their F_1 hybrids

Compared with the Martonvásári 4 parent, the frost resistance of the F_1 population showed no deviation at –12°C or –14°C, but was significantly lower at –16 and –18°C. The percentage survival of the F_1 population was significantly higher than that of Martonvásári 10 at –14°C and –16°C, while freezing at –18°C gave no significant deviation.

Discussion

Studies were made on how the overwintering of winter cereals is influenced by the environmental factors shown by predictions on global climatic changes to be likely to affect Hungary. There may be a reduction in winter precipitation, the winters may be warmer and an increase in atmospheric CO_2 concentration is to be expected (Czelnai, 1994).

The results of these experiments confirm previous findings that not all changes are necessarily unfavourable for the living world (Thompson and Woodward, 1994; Kimball, 1983). However, the development and biomass production of the plants are determined by the joint effect of the changes. A rise in the winter temperature and an increase in the atmospheric CO_2 concentration will have a favourable influence on the overwintering of winter cereals. In milder winters, on the other hand, conditions favour the survival of pathogens and pests (Kozár et al., 1996), while the expected reduction in the quantity of precipitation will also have an unfavourable effect. This could mean that the increase in the number of plants overwintering will be offset by yield losses for the above reasons.

The experimental data demonstrate that the overwintering of winter wheat varieties is influenced not only by genetic determination and environmental factors, but also by biotic stress factors, i.e. bunt infection.

No references concerning the joint effect of biotic (bunt infection) and abiotic (frost) stress factors under controlled conditions were found in the literature. In the present experiments a close ($r = 0.890$) correlation was demonstrated between the bunt susceptibility of the tested Martonvásár varieties and the increase in frost kill due to infection, indicating that the frost killed plant rate for susceptible varieties was greater as the result of infection than that of less susceptible varieties. It could be that the chemical substances involved in cold hardening (e.g. sugars, carbohydrates, lipid composition of the membranes) are used up by the pathogen during the development of the disease, leading, in the case of more severe infection, to greater frost sensitivity due to a reduction in the concentration of the cell solution.

In an earlier complete diallel analysis involving 12 winter wheat varieties frost resistance was found to be partially dominant (Veisz et al., 1997), in agreement with the results reported by Eunus et al. (1962) and Rohde and Pulham (1960). The data indicated that when freezing was carried out at –15°C frost resistance was dominant over frost sensitivity (Veisz et al., 1997). This result was contradicted by some of the literary data, since in experiments carried out by Puchkov and Zhirov (1978) and Sutka (1981) the dominant genes led to poorer frost resistance and the recessive genes to better resistance.

It is quite clear that dominance relations also depend on what genotypes are examined and on which alleles are in the majority in the varieties tested. In the present experiment in both frost-resistant and frost-sensitive variants frost resistance was dominant at relatively mild freezing temperatures, while a reduction in the testing temperature led to a reversal of the dominance, so that frost sensitivity became dominant.

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INHERITANCE OF FROST RESISTANCE IN WHEAT (*TRITICUM AESTIVUM* L.)

J. SUTKA[1], G. GALIBA[1] and J.W. SNAPE[2]

[1]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, HUNGARY
[2]JOHN INNES CENTRE, COLNEY, NORWICH, NORFOLK, NR4 7UH, UK

Frost is one of the most severe abiotic stress factors limiting wheat plant growth, productivity and distribution. Low-temperature hardening allows winter wheat to protect critical cell structures and physiological processes during periods of freezing temperature.

Over the last 50 years a large number of different methods have been used for studying frost resistance. Precise genetic studies can be carried out under artificial conditions, where the climatic factors of growth, hardening, freezing and regrowth after freezing are controlled. The Martonvásár phytotron provides excellent facilities for accurate studies on frost resistance.

A detailed study of the genetic control of frost resistance in wheat was started in the Martonvásár phytotron in the early 1970s. Diallel crosses involving six and ten wheat varieties were tested for frost resistance. The results indicated that frost resistance was controlled mainly by additive genetic effects. Non-additive genetic variation was present as dominance only. Generally the dominant genes lowered frost resistance and the recessive ones raised it. Several cytogenetic studies have been conducted using monosomic and substitution analysis. The results showed that at least 10 of the 21 pairs of chromosomes were involved in the control of frost resistance. Chromosomes 5A and 5D were found to have the largest effect on frost hardiness. Genes on these chromosomes are expressed not only at plant level but also at tissue culture level.

QTL (Quantitative Trait Loci) analysis revealed that a locus of major effect influencing vernalisation response (*Vrn1*) co-segregated with three RFLP (Restriction Fragment Length Polymorphism) markers (*Xpsr426, Xwg644, Xcdo504*) was closely linked to the location of frost resistance gene *Fr1* on the long arm of chromosome 5A. Genetic analysis is now being conducted to study the location of frost resistance gene *Fr2* using the molecular marker systems RFLP and AFLP. Preliminary results indicate that *Fr2* is located on the long arm of chromosome 5D, and is linked to, but separable from vernalisation gene *Vrn3*.

Key words: wheat, frost resistance, inheritance, diallel analysis, chromosomal location, genetic mapping

Introduction

Frost is one of the environmental abiotic stress factors reducing wheat yield significantly in many countries. If wheat seedlings are frost-resistant, it means that they can survive the frost effect without any considerable damage.

The study of the inheritance of frost resistance was started early in this century by Nilsson-Ehle (1912), who crossed two winter wheat varieties intermediate for winter hardiness and found transgressive segregation for the character. Over the last 50 years a large number of different methods have been used for studying frost resistance. Most of these experiments were carried out under field conditions for the study of winter hardiness. Precise genetic studies can be carried out under artificial conditions, where the climatic factors of growth, hardening, freezing and regrowth after freezing are controlled. The Martonvásár phytotron provides excellent facilities for accurate studies on frost resistance (Tischner et al., 1997).

A detailed study of the genetic control of frost resistance in wheat was started in the Martonvásár phytotron in the early 1970s. Three periods of study can be differentiated in chronological order. In the first period gene action and interaction were investigated using quantitative genetic analysis. In the second period genes controlling frost resistance were located on chromosomes by means of classical cytogenetic methods. In the third period gene mapping was performed using molecular markers. Various genetic and cytogenetic materials, including diallel hybrids, monosomic sets and F_2 hybrids, substitution series, chromosome recombinations, etc. were used for the frost test.

Germinated wheat seeds for the artificial freezing test were sown randomly in wooden boxes measuring 42×31×18 cm. The growing medium was a 2:2:1 mixture of garden soils, humus mixture and sand. Thirty rows, each consisting of five germinated seeds, were sown in each box. Each row represented a single line and the 30 combinations found in each box were taken as one replication. The special autumn-type climatic programme elaborated for studying the genetic control of frost resistance is relatively simple and thus easily reproducible in other research institutes (Sutka, 1981; Tischner et al., 1997). Frost resistance was assessed in terms of regrowth on a 0 (dead) to 5 (undamaged) scale and also as percentage survival.

Quantitative genetic analysis

The inheritance of frost resistance in wheat was studied in detail by Gullord (1975) and Puchkov and Zhirov (1978). Frost resistance was found to be controlled mainly by an additive-dominance system.

A six-parental cross including reciprocals was carried out, and F_1 hybrids and their parents were used for freezing tests under controlled conditions in the Martonvásár phytotron (Sutka, 1981). Both the general combining ability (GCA) and the specific combining ability (SCA) were significant, indicating additive and non-additive gene action in the inheritance of frost resistance. The high GCA:SCA ratio revealed a preponderance of additive genetic variance. No significant reciprocal differences were found between the reciprocal crosses. The variance/covariance graphical analysis indicated the partial dominance of frost sensitivity. Frost-sensitive varieties had the largest number of dominant genes, while frost-resistant varieties had the highest proportion of recessive genes. The magnitude of the additive component of variation was higher than that of the dominance component, and the overall measure of the degree of dominance was smaller than one, so average dominance is incomplete. The increasing and decreasing alleles were not equally frequent at all loci. The values of narrow and broad heritability were 81.10% and 97.55%, respectively.

These results were confirmed by the analysis of another diallel cross involving ten wheat varieties (Sutka, 1984). It was concluded that frost resistance was a quantitative character determined by several genes.

Location of genes on chromosomes

Various wheat monosomics and substitutions were used to determine the location on the chromosomes of genes responsible for frost resistance and winter hardiness. If the differences between varieties for frost resistance are large, monosomic analysis is suitable for the location of particular genes (Puchkov and Zhirov, 1978; Sutka and Rajki, 1979; Sutka, 1981; Rigin and Barashkova, 1984; Sutka and Kovács, 1985).

Intervarietal chromosome substitutions provide one of the best means of studying the genetic control of frost resistance. In our experiments the survival of the Chinese Spring/Cheyenne substitutions was tested under artificial and natural conditions (Sutka, 1981; Sutka et al., 1986). In each of the substitution lines one pair of chromosomes from the Chinese Spring variety was replaced by the corresponding pair of chromosomes from the frost-resistant variety Cheyenne. Freezing tests in phytotron chambers confirmed earlier observations which indicated that the chromosomes of the 5th homoeologous group of Cheyenne carry major genes controlling frost resistance (Cahalan and Law, 1979; Roberts, 1986). When comparing phytotronic frost testing and nursery winter hardiness it can be seen that chromosomes of the 5th homoeologous group and the 2B and 4B chromosomes play an important role in both environments (Table 1). Considerable differences were also observed. In the phytotron, for instance, chromosome 6A reduced frost resistance compared to the recipient Chinese Spring, while it increased winter hardiness in the field. The effect of chromosomes 7A and 4D is only manifested during artificial testing, not in the field. We concluded that at least 10 of the 21 pairs of chromosomes are involved in the control of frost resistance and winter hardiness. Chromosomes 5A and 5D appear to carry major genes.

The frost resistance of Chinese Spring/Cheyenne substitution lines was also studied in callus culture. Callus cultures were induced from 12–14-day-old immature embryos. After four weeks the calli were subcultured and maintained for an additional four weeks. The calli were cultured at a temperature of 26°C with a 16 h day and an illumination of 1500 lux. After a 6-week hardening period, freezing was conducted at different temperatures. The calli were frozen in Petri dishes (10 cm in diameter). After thawing the viability of the calli was tested using the triphenyltetrazolium chloride (TTC) method. There was a

Table 1
Frost resistance and winter hardiness of Chinese Spring/Cheyenne chromosome substitutions under phytotronic and field conditions (percentage survival)

Chromosome substitution	Phytotron (–11°C)	Field
1A	59	17
2A	45	12
3A	39	25
4A	58	25
5A	94***	71***
6A	31*	56***
7A	75***	22
1B	63	19
2B	74***	31*
3B	62	19
4B	74***	40***
5B	73**	47***
6B	65*	30
7B	48	19
1D	61	21
2D	43	28
3D	36	23
4D	75***	17
5D	93***	41***
6D	59	31*
7D	37	22
Chinese Spring (recipient)	49	19
Cheyenne (donor)	100***	70***

*, **, *** P=0.05, P=0.01 and P=0.001, respectively

significant difference between the survival rates of Chinese Spring and Cheyenne. At a temperature of –11°C the viability of Chinese Spring calli rapidly decreased from 100% to 25%. At –13°C only a few cells or cell aggregates survived. On the other hand, Cheyenne calli tolerated both the –11°C and –13°C treatments giving 75% survival rates, but –15°C proved lethal. The frost resistance of two substitution lines, 5A and 5D, was significantly different from that of Chinese Spring at both –11°C and –13°C. Similar results were obtained for Cheyenne. The results of this study indicated that genes on chromosomes 5A and 5D are expressed not only at plant level but also at tissue culture level (Galiba and Sutka, 1988).

Genetic mapping

The location of the gene(s) responsible for frost tolerance on chromosome 5A was studied using recombinant substitution lines from a cross between the substitution line Hobbit (*Triticum spelta* 5A) and Hobbit. In this sample of recombinant lines the locus for frost resistance, designated *Fr1* (*Frost*), was completely linked to the locus *Vrn1* (*Vernalisation*). The results can be explained by the pleiotropic action of the *Vrn1* locus or by close genetic linkage between *Vrn1* and *Fr1* (Sutka and Snape, 1989). The locus involved in the control of cold hardiness on chromosome 5A of wheat was also identified by Roberts (1990).

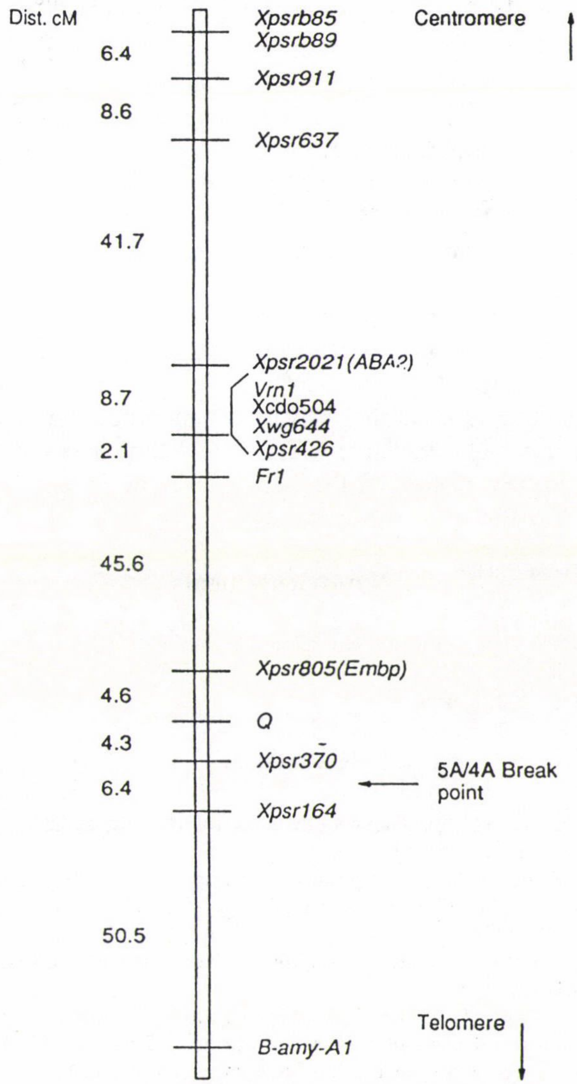

Fig. 1. Genetic map of wheat chromosome arm 5AL showing the location of the *Vrn1* and *Fr1* loci

A population of recombinant substitution lines was developed from the cross between a frost-sensitive, vernalization-insensitive substitution line, Chinese Spring (*Triticum spelta* 5A), and a frost-tolerant, vernalisation-sensitive line, Chinese Spring (Cheyenne 5A), and used to map the genes *Vrn1* and *Fr1*, controlling vernalization requirement and frost resistance, respectively, relative to RFLP markers located on this chromosome (Sutka et al., 1994). The *Vrn1* and *Fr1* loci were located closely linked on the distal portion of the long arm of 5AL, but contrary to previous observations, recombination between them was found (Fig. 1). Three RFLP markers, *Xpsr426, Xcdo504* and *Xwg644,* were tightly linked to both (Galiba et al., 1995).

The analysis is now being extended to wheat chromosome 5D to map the presumptive locus, *Fr2,* using the molecular marker systems RFLP and AFLP. Preliminary analysis indicates that *Fr2* is located on the long arm of chromosome 5D, is homoeologous to *Fr1*, and linked to, but separable from, *Vrn3*. These loci form part of an adapted gene complex which has pleiotropic effects on a range of characters associated with stress resistance (Snape et al., 1997).

Recently, the distribution of the genes in chromosome group 5 of wheat was studied using chromosome deletion lines by comparing physical maps with the genetic linkage maps. More than 60% of the long-arm markers of 5A were present in three major clusters that physically encompassed <18% of the arm. Based on our preliminary results it seems likely that genes *Vrn1* and *Fr1* are flanked by two probes mapped in the gene cluster at FL 0.75 on chromosome 5AL (Gill et al., 1996).

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MAPPING FROST TOLERANCE LOCI IN WHEAT AND COMPARATIVE MAPPING WITH OTHER CEREALS

J. W. SNAPE[1], A. SEMIKHODSKII[1], L. FISH[1] R. N. SARMA[1], S. A. QUARRIE[1], G. GALIBA[2] and J. SUTKA[2]

[1]JOHN INNES CENTRE, NORWICH RESEARCH PARK, COLNEY, NORWICH, NR4 7UJ, UK
[2]AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, H-2462 MARTONVÁSÁR, HUNGARY

Identifying and mapping the individual genes controlling frost resistance in wheat is difficult because effects are quantitative in nature and require precise and repeatable experimental conditions. Nevertheless, through the use of precise genetic stocks combined with RFLP markers, particularly recombinant substitution lines, progress has been made. In this way the *Fr1* gene on chromosome 5A was mapped. This analysis has now been extended to chromosome 5D, to map the locus, *Fr2*, whose chromosomal location was previously indicated by chromosome assay. However, the very low levels of RFLP for this chromosome has made mapping difficult and progress has required the use of the new molecular marker system, AFLP to fill gaps between 'anchor' RFLPs. Analysis confirms that *Fr2* is located on the long arm of chromosome 5D, is homoeologous to *Fr1*, and linked, but separable, from *Vrn3*. A clear homoeologous relationship to loci on chromosome 7 (5H) of barley, and 5R of rye has been shown by cross-referencing maps containing common RFLP marker loci. This comparative genetic analysis is now being extended to other species, particularly rice, so that a strategy for positional cloning of the presumptive loci can be developed.

Key words: wheat, mapping, frost tolerance, vernalization

Introduction

Tolerance of low temperatures is a major component of the adaptation of wheat to the northern and central European environment. Wheat varieties are well known to differ in their responses to low temperatures and numerous studies have shown that the genetic control of frost tolerance is complex and can be regarded as a polygenic trait. Nevertheless, studies using precise genetic stocks, particularly single chromosome substitution lines, have identified specific chromosomes that carry genes mediating large effects. In particular, the homoeologous group 5 chromosomes, especially 5A and 5D, have featured consistently as chromosomes carrying major components of frost response (Sutka, 1981; Sutka and Snape, 1989).

Detailed genetic analysis of a population of recombinant substitution lines for chromosome 5A using molecular markers mapped a major gene, designated *Fr1*, on the long arm and showed that this is very closely linked to, but separable from, the gene *Vrn1* mediating vernalization response (Galiba et al., 1995). It is of great interest to extend these studies to the presumed homoeologous locus on chromosome 5D and to predict and test for the presence

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of homoeologous loci in related cereal species. In this paper we present data on mapping of *Fr2* on chromosome 5D and show its probable homoeology to *Fr1* and frost tolerance QTL identified in other species.

Molecular markers, maps and mapping strategies for frost resistance loci in wheat

The development of molecular marker systems in wheat, as in other species, has revolutionised the ability to develop comprehensive genetic maps, and hence the power to carry out detailed genetic analysis for major genes and quantitative trait loci (QTL). The requirements for mapping major genes and QTL for frost tolerance are (i) to have a suitable mapping population, (ii) to have appropriate molecular marker technologies, (iii) to be able to measure the trait using appropriate experimental conditions, (iv) to have available suitable statistical methodologies to combine the trait and molecular marker data. All of these were available for the present study:

(i) The mapping population consisted of a recombinant substitution line population developed from the cross between Chinese Spring (CS) and the single chromosome substitution line CS (Cheyenne 5D). CS is sensitive to frost and the substitution line relatively resistant (Sutka, 1981). The recombinant lines were developed using the technique described by Law (1996).

(ii) Most genetic maps of wheat have been based on RFLP systems, and the first complete genetic map was developed by Gale et al. (1994) based on such markers. Nevertheless, levels of RFLP are generally low in wheat and chromosome 5D is particularly difficult (Snape et al., 1994; Cadalen et al., 1997). Therefore, alternative strategies are needed to map in adapted crosses when this chromosome is being studied. Fortunately, recent developments in marker technologies are allowing this to be possible, in particular, the development of Amplified Fragment Length Polymorphisms (AFLP) (Vos et al., 1995) technologies. Thus, the strategy developed to map here was to use RFLP probes previously located on the homoeologous group 5 chromosomes to search for polymorphisms to provide 'anchor markers' for the map and then to use AFLP markers to provide greater resolution.

(iii) The frost resistance of each of the recombinant substitution lines and their parents was carried out using the facilities of the Martonvásár Phytotron, as described by Galiba et al. (1995). In addition, since chromosome 5D is known to control flowering time through the vernalization response locus *Vrn3*, the flowering time of each line was characterised in a growth chamber under a regime of 16 h day – 8 h night at temperatures of 22 and 15°C, respectively.

(iv) JoinMap (Stam, 1993) was used to develop the genetic map of chromosome 5D based on the segregational patterns of the RFLP and AFLP markers and the classification of individual recombinant substitution lines for flowering time and frost tolerance. Additionally, since the frost tolerance data did not allow unambiguous classification of all genotypes into resistant and susceptible categories, different QTL methodologies, those of Interval Mapping (Lander and Botstein, 1989) and Marker Regression (Kearsey and Hyne, 1994) were applied using the computer package JAQC developed by Kearsey et al. (1997).

Location of *Vrn3* and *Fr2* on chromosome 5D

All the recombinant substitution lines were unambiguously characterized for flowering time under controlled environment conditions. The population showed a bimodal distribution based around the parental scores (Fig. 1), characteristic of segregation for a single gene with *Vrn3* (spring type) and *vrn3* (winter type) alleles at the *Vrn3* locus. Frost tolerance was scored as % survival after freezing at –11°C and –13°C, and although the parents showed a distinct difference, no clear segregational patterns were apparent in the recombinant population (Fig. 1). Thus, a complete, unambiguous classification of each genotype as resistant or susceptible to the low temperatures was difficult. Nevertheless, from the distribution of scores there is a clear relationship between *Vrn3* and the presumed frost resistance locus *Fr2*, indicating linkage between these loci. However, there also appear to be genotypes which are early, yet frost resistant, and genotypes that are late but frost sensitive, showing that linkage rather than pleiotropy is responsible for the relationship.

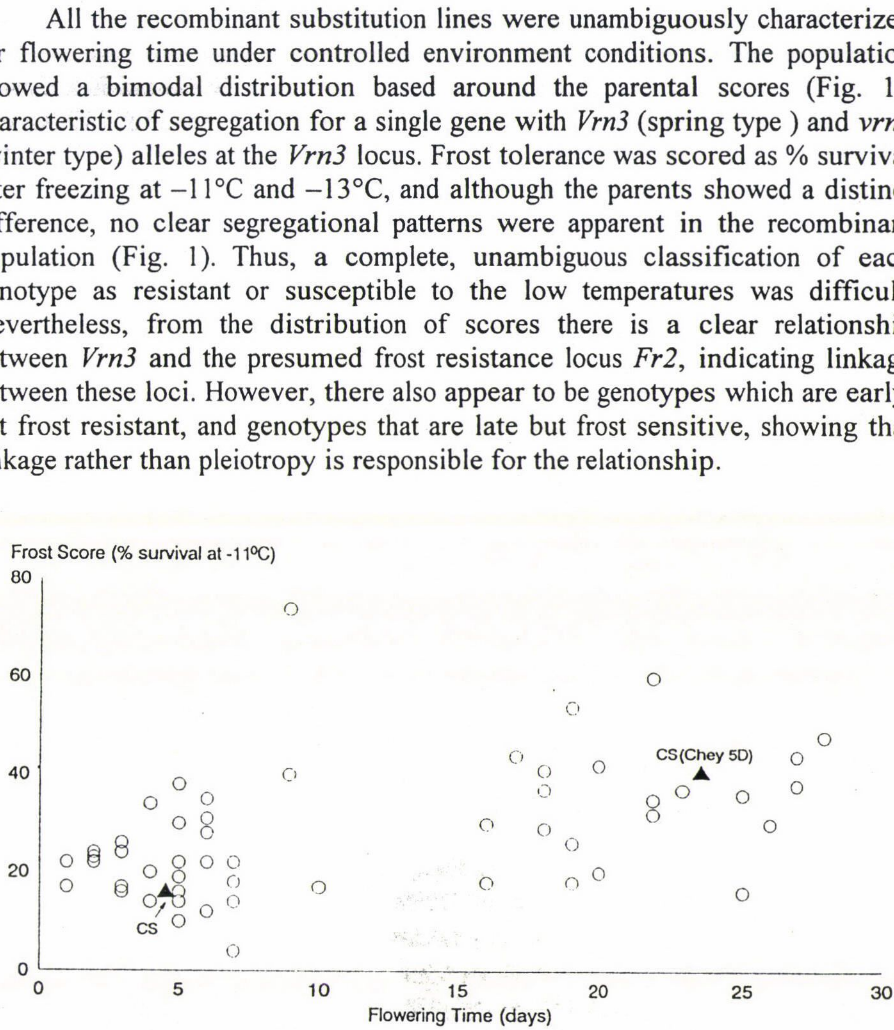

Fig. 1. Relationship between flowering time and frost resistance score (% survival at –11°C) for the population of recombinant substitution lines from the CS × CS (Cheyenne 5D) cross

To map *Vrn3* and *Fr2* on the long arm of 5D RFLP and AFLP systems were used. First, RFLP probes known to map to chromosomes of homoeologous group 5 of wheat were tested for polymorphism between the parents of the mapping population, CS and CS (Cheyenne 5D). These consisted of a range of genomic and cDNA clones from wheat, barley and oats (Galiba et al., 1995). However, only two probes, WG232 and PSR567, both located on the long arm of the group 5 chromosomes, revealed polymorphisms suitable for mapping, and each was hybridized to each of the recombinant lines for genotypic classification. Because of the paucity of RFLP, the AFLP technique was applied to DNA of the recombinants. Seven primer combinations were used and these jointly revealed eight scorable polymorphisms. Based on this marker information associations between the markers and the flowering time and frost tolerance data were tested using ANOVA and QTL mapping methodologies. The two RFLPs and four of the AFLPs showed a significant association, confirming the location of *Vrn3* and *Fr2* on the long arm of 5D. Based on these analyses a map of the long arm of chromosome 5D was constructed for this CS × CS (Cheyenne 5D) population containing *Vrn3*, *Fr2,* the two RFLPs and three of the AFLPs (Fig. 2). This map confirms that *Vrn3* and *Fr2* are linked, but separable loci, and that *Fr2* appears to be further away, genetically, from *Vrn3* than *Vrn1* is from *Fr1*.

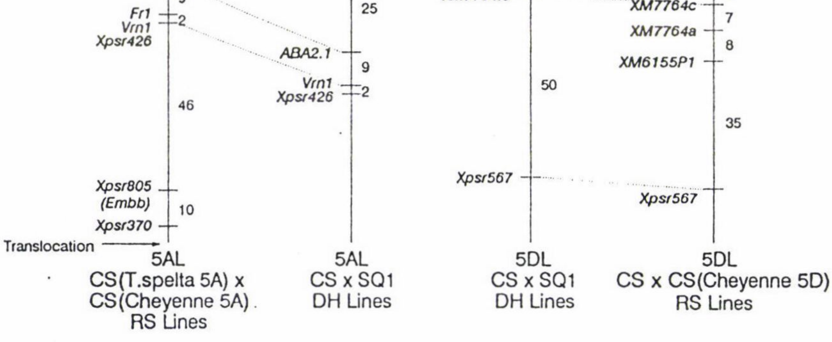

Fig. 2. Wheat comparative maps for chromosomes 5A and 5D

Comparative mapping of vernalization and frost tolerance loci

Based on data from Galiba et al. (1995), Semikhodskii et al. (1997) and the present experiments, comparative maps for chromosomes 5A and 5D can be developed to assess whether *Vrn1* and *Vrn3*, on the one hand, and *Fr1* and *Fr2*, on the other, are indeed homoeologous. These are presented in Fig. 2. From the positions of cross-hybridizing RFLP probes, it is clear that the loci map in homoeologous regions of the long arms of 5A and 5D and, therefore, are undoubtedly homoeologous. This analysis fits in with other comparative studies of this region where *Sh2* on chromosome 5H of barley, and *Sp1* on chromosome 5R of rye are homoeologous loci to *Vrn1/Vrn3* (Galiba et al., 1995; Snape et al., 1995), and *Fr1* and *Fr2* are probably homoeologous to a frost tolerance QTL detected on barley 5H by Pan et al. (1994).

Experiments are now underway to extend this analysis further to other cereal species particularly rice, using a mapping population developed at the John Innes Centre between the indica variety IR20 and the javanica variety 63–83. Preliminary analysis using cross-hybridizing wheat and rice cDNA probes suggests that a homoeologous region containing a flowering time QTL exists on rice chromosome 3 (Sarma and Snape, unpublished). This latter finding is exciting in that it suggests that the region may contain loci homoeologous to Vrn and Fr loci which could be isolated by positional cloning using rice YACs following the strategy proposed by Moore et al. (1993).

Discussion

By using precise genetic stocks combined with molecular marker technologies it has been possible to locate QTL responsible for the major components of frost resistance in wheat on chromosomes 5A and 5D. Although 5A seems to be the more important effect, nevertheless, homoeologous variation is present on 5D. Further analysis of chromosome 5B may reveal a further locus, *Fr3*. Combining superior frost resistance from these loci in a single genotype should be possible by marker-assisted selection, and could provide an important route for plant breeders to improve frost resistance in wheat.

The mechanism behind the frost resistance imparted by these loci remains obscure at present. However, further analysis of the recombinants for the long arm of 5A has shown that this region carries genes controlling soluble carbohydrate production in response to cold, and other aspects of stress responses (Galiba et al., 1997). This indicates that either this region contains a complex of genes, or all these effects are the pleiotropic actions of a few genes. It will require the cloning of the genes in this region and their molecular and biochemical characterization before the mode of actions of the genes can be properly described.

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GENES AND GENE PRODUCTS REGULATED BY LOW TEMPERATURE TO IMPROVE WINTER HARDINESS OF BARLEY

L. CATTIVELLI, C. CROSATTI, M. GROSSI, P. BALDI, E. GIORNI, F. RIZZA and A. M. STANCA

EXPERIMENTAL INSTITUTE FOR CEREAL RESEARCH, VIA S.PROTASO 302, I-29017 FIORENZUOLA D'ARDA, ITALY

Frost resistance in barley is important because barley growing has been shifting for a number of years from spring to autumn sowing as winter varieties are higher-yielding than spring cultivars. It is, however, a known fact that the winter barley varieties are less hardy than winter wheat, rye and triticale. Studies of the physiological mechanisms controlling the adaptation of barley to stress conditions have focused on two areas: **a**) metabolic changes associated with cold adaptation and **b**) modification of gene expression and cold-regulated (COR) genes. Changes in free proline, ABA, water-soluble carbohydrates and free fatty acids were measured to assess their involvement in cold-acclimation and to explain the different frost-resistant capacities of contrasting genotypes. In particular the ABA content after two days of hardening and the free fatty acid composition were different when using a frost-resistant and a frost-sensitive genotype.

We are interested in studying the molecular basis of the cold acclimation response enabling barley to survive under stress. Several genes regulated by low temperatures and sometimes by drought have been isolated from the barley genome. In this review the most significant results of our recent work will be presented and discussed.

Key words: barley, hardening, cold stress

Introduction

A great deal of attention has recently been focused on many biochemical and molecular responses of barley to environmental stresses in an effort to understand their links to resistance under field conditions.

The optimum development of barley over its life cycle depends on a number of environmental stress factors that can prevent the plant from expressing its maximum genetic potential. Severe grain losses are often caused by high or low temperatures, drought and soil anomalies. The responses elicited from the plant by these stresses, when not lethal, include alterations in the processes of photosynthesis, respiration and hormonal regulation through the development of specific, adaptive defense systems and mechanisms that are genetically controlled. The duration of the stress and the plant growth stage at the onset of stress in turn affect yield. One can also find differing reactions as to plant susceptibility to adverse conditions. Thus, genetic variability plays a primary role in determining positive adaptation to environmental stresses and, hence, in supporting the spread of various barley genotypes to extreme climatic conditions (Cattivelli et al., 1994).

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Frost resistance in barley is an important agronomic character because barley growing has been shifting for a number of years from spring to autumn sowing as winter varieties are higher-yielding than spring cultivars. The tolerance of winter cereals to low temperatures depends on the physiological process known as hardening or acclimation that occurs when plants are exposed to temperatures ranging from 0 to +5°C. Cold acclimation involves a number of biochemical and molecular changes that enable certain plant species to withstand periods of very intense cold (Cattivelli and Bartels, 1989; 1992; Stanca et al., 1996).

Expression of cold regulated (COR) genes

Frost-resistant plant cells show variations in some fundamental biochemical pathways (e.g. sugar and fatty acid metabolisms (Murelli et al., 1995) as well as in the properties of the membranes (Wolter et al., 1992) and of the photosynthetic apparatus (Huner et al., 1993). Studies undertaken to understand the molecular processes controlling such variations have led to the isolation of many cold-regulated (COR) genes. In barley probably more than 20 cDNA clones whose expression is affected by low temperatures have been isolated (Cattivelli and Bartels, 1990; Dunn et al., 1990; Dunn et al., 1991; Goddard et al., 1993; Hughes et al., 1992). Most of these genes are expressed only at low temperatures, while only few are involved also in plant response to other environmental stress situations (e.g. drought). The expression of the COR genes is controlled by different regulation mechanisms: some COR sequences have been shown to be transcriptionally regulated, while others are controlled by post-transcription mechanisms (Dunn et al., 1994). The analysis of the expression pattern reveals that the COR mRNAs reach their steady state within 2–3 days after exposure to cold, while when the plants are moved from low temperatures to 20°C the level of COR mRNAs drops in a few hours (Cattivelli and Bartels, 1990). Despite the fact that many barley COR genes have been sequenced, important homologies have only been found for a few of them. The clone *blt4* shows an interesting homology with genes coding for lipid transfer proteins (Hughes et al., 1992), a second barley COR gene (*blt63*) codes for a translation elongation factor 1α (Dunn et al., 1993), while the *blt801* gene encodes a protein with single strand nucleic acid binding activity (Dunn et al., 1996). The barley cDNA clone *cdr29* is homologous to a class of mRNA regulated by low temperature and drought stress and shows a sequence similarity with animal and yeast genes coding for peroxisomal acyl coenzyme-A oxidase, an enzyme involved in the oxidation of fatty acids (Grossi et al., 1995). A comparison of this sequence with those present in the data bank showed, both at the nucleotide and at the amino acid level, a region of high homology with known sequences coding for peroxisomal acyl coenzyme A oxidase of rat, human, *Caenorhabditis elegans* and *Candida*. The sequence comparison showed the existence of a highly conserved domain through all the sequences.

Accumulation of COR proteins

The cold-regulated protein of 14 kDa (COR14) is a polypeptide accumulated under low temperature conditions in the chloroplasts of barley leaves (Crosatti et al., 1995). The corresponding gene, *pt59* (Cattivelli and Bartels, 1990) is expressed in leaves of barley, as well as of other related cereals, during plant exposure to low temperatures. The COR14 antibody cross-reacts with two proteins with slightly different relative molecular weights around the marker of 14.4 kDa refered to as COR14a and COR14b (high and low relative molecular weights, respectively). The accumulation of COR14 proteins was monitored throughout hardening and subsequent dehardening in leaves of barley plants. COR14 was detected after about 7 days exposure to low temperatures and gradually accumulated over a period of up to 4 weeks. When the plants were subsequently transferred to 20°C, COR14 persisted for at least 6 days, a finding in contrast with a previous study showing that the messengers corresponding to *pt59* disappeared a few hours after the temperature was raised (Cattivelli and Bartels, 1990). Analysis of the proteins in the subcellular fractions indicates that COR14 is accumulated in the stroma fraction of the chloroplasts.

Light-dependent accumulation of COR 14 proteins

The chloroplast localization suggested a possible involvement of light in the regulation of COR14 accumulation. Therefore, the effect of light on the accumulation of chloroplast-localized COR proteins was tested by comparing plants grown and hardened under standard and non-standard photoperiods. While the transcription of the *pt59* gene and the translation of the corresponding mRNA occur only at low temperature, light markedly stimulates gene expression and is needed for protein accumulation. The regulation of gene *pt59* does not require the concomitant presence of both factors: indeed plants only need to be exposed to light for a short time (5 min) to induce *pt59*-corresponding mRNAs when the temperature is low. This fact indicates that the expression of *pt59* mRNA is mediated by a light-regulated factor and that even a 5-min light exposure is enough to induce this factor to such an extent as to normalize gene expression. A brief exposure of plants to light ensures normal *pt59* expression but is not enough to determine normal COR14 accumulation. This suggests that the presence of light, or of chloroplast activity, influences COR14 accumulation (Crosatti et al., 1995).

The accumulation of COR proteins is associated with the developing of cold hardening

Since the function of many COR proteins is still unknown, a possible approach in order to demonstrate the importance of the molecular response to low temperatures, is to look for a correlation between the amount of mRNAs or proteins and the degree of frost resistance. For instance, an analysis of the expression patterns of COR genes has sometimes shown a positive correlation between the accumulation of COR transcripts and the degree of cold resistance in some barley cultivars (Dunn et al., 1990), although in other cases the same approach did not allow cold-susceptible and cold-resistant cultivars of barley to be distinguished *via* COR-transcript expression (Dunn et al., 1991) or COR protein accumulation (Crosatti et al., 1994).

A clear correlation between the degree of freezing tolerance and the accumulation of a specific COR protein has been found for the wheat protein WCS120. The corresponding antibody not only discriminates between frost-resistant and frost-susceptible wheat cultivars but, because it also recognizes the WCS120 homologous protein of related cereals, it has been proposed as a marker to select for freezing resistance in all gramineae species (Houde et al., 1992).

The physiological evidence that frost-resistant cultivars have faster hardening and slower dehardening when compared with frost-susceptible genotypes (Rizza et al., 1994) suggested that the cold acclimation process may have different threshold induction temperatures in spring and winter barley. We have tested this hypothesis by using the antibody raised against the COR14 proteins and 30 barley cultivars previously evaluated for their level of frost resistance (Crosatti et al., 1996). All these cultivars were cold-acclimated for 7 days at constant temperature (6°, 8° or 10°C) and subjected to western analysis. When the frost-resistant winter cultivars were compared with the frost-sensitive spring ones a clear difference was detected: at a temperature of 8°C COR14a was accumulated in the former, but not in the latter cultivars indicating that, in barley, a higher degree of frost resistance is associated with a higher threshold induction temperature for the accumulation of COR14a protein.

In a *H. spontaneum* collection (Nevo et al., 1979) a clear polymorphism was found for the corresponding COR proteins. While some accessions showed the same COR pattern as cultivated barley, in 38 out of 61 *H. spontaneum* accessions examined, the COR14 antibody cross-reacted with an additional cold induced protein with a relative molecular weight of about 24 kDa (COR24). The accumulation of COR24 was associated with the absence of COR14b. COR14a was found in all samples examined. A large variation in the amount of COR24 accumulated by *H. spontaneum* plants under low temperatures has also been detected. Most of the accessions expressing COR24 accumulated this protein at a high level, but there were also a few accessions expressing COR24 at very low level.

The COR14/24 polymorphism did not affect either the expression kinetics or the subcellular localization of the proteins. Indeed, the cold-triggered accumulation kinetics of COR14 and COR24 were the same and both proteins are localized in the stroma of the chloroplasts (Crosatti et al., 1996). The common expression pattern and subcellular localization of COR14b and COR24 together with their high immunological relationship suggest that probably these two proteins have the same, if still unknown, function. *H. spontaneum* is polymorphic for COR24 and COR14b in highland regions exposed occasionally or routinely to frost. By contrast COR24 is monomorphic and COR14b is absent in warm desert and mild, usually frost-free coastal plain populations. Thus, the polymorphism of COR24 and COR14b may be climatically adapted.

These results provide a good example of how molecular markers can be either a breeding tool for selecting superior genotypes with increased frost resistance or useful for studying plant adaptation to different thermal environments.

Expression of dehydrin genes during cold acclimation

The ability of plants to survive under freezing temperatures also involves the capacity to withstand reduced water availability. Therefore it is not surprising that several genes involved in the cold response are also expressed during dehydration and vice versa. Of course, a number of modifications of gene expression specific for cold or for drought response have also been observed (Grossi et al., 1992). When barley plants are subjected to a reduction in water availability the typical molecular response leads to the accumulation of a class of dehydration-related proteins called LEA or dehydrins. These polypeptides are characterized by a consensus 15-amino acid domain rich in lysine (EKKGIMDKIKEKLPG) that may be present several times in the sequence. In many dehydrins an additional characteristic domain rich in serine is present adjacent to the lysine-rich one (Chandler and Robertson, 1994). While most of the dehydrin genes are expressed under dehydration or by exogenous abscisic acid (ABA) application, only few of them are induced during cold-acclimation in barley. Crosatti et al. (1994) have described a COR protein of 75 kDa (COR 75) representing the most abundant COR protein in barley. COR 75 was shown to be moderately induced by exogenous ABA application as well as by drought stress. By using a polyclonal antibody COR 75 was found to be immunologically related to the high-molecular-weight COR proteins of wheat. After purification and N-terminal microsequence this protein was found to be DHN5 (F. Rizza, unpublished results).

A feature common to all dehydrins is their induction by ABA and indeed promoter sequences that mediate the ABA responsive transcription have been described for several dehydrin genes (Chandler and Robertson, 1994). Nevertheless, we have found that in barley there are also genes coding for proteins very similar to the dehydrins whose expression is independent of ABA. One example is the cDNA clone *paf93:* this clone was originally identified as induced by low temperatures (Cattivelli and Bartels, 1990), although recently its involvement in the dehydration response has also been described (Grossi et al., 1995). During the early stage of dehydration, the *paf93* mRNAs are expressed before the induction of known ABA regulated genes such as dehydrins and when only a small increase occurs in ABA content. The protein sequence deduced from the cDNA clone *paf93* shows a serine cluster and four lysine-rich domains, demonstrating its homology with the dehydrin proteins. A comparison of the amino acid sequence of *paf93* with those in the Swiss-Prot data bank indicates that the most similar sequences are in the cotton LEA protein D-11 (Baker et al., 1988) and in the *Arabidopsis* COR47 protein (Gilmour et al., 1992).

Conclusions

Cloned genes provide tools for the understanding of the molecular mechanisms involved in the adaptation of plants to limited environments. It is generally accepted that the expression of stress-related genes is an essential part of plant adaptation processes, although for many genes direct evidence of their function is still lacking. The use of transgenic plants expressing sense and antisense constructs is expected to be the most powerful tool in understanding the role of cloned genes. Unfortunately the transformation of barley is still not routine. Nevertheless, in model plant species such as tobacco and *Arabidopsis* new genotypes with an improved adaptation to stress environments have already been produced.

New approaches to breeding strategies in the cold resistance of barley are expected to emerge from recent studies in the molecular biology of cold inducible genes, an aspect that has aroused keen interest.

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INDUCTION OF FREEZING TOLERANCE AND SNOW MOULD RESISTANCE IN WINTER WHEAT: BIOCHEMICAL AND MOLECULAR PERSPECTIVES

A. LAROCHE[1], D. A. GAUDET[1], P. AUDY[1], M. M. FRICK[1] and J. MULLIN[2]

[1]AGRICULTURE AND AGRI-FOOD CANADA RESEARCH CENTRE, P.O. BOX 3000, LETHBRIDGE, ALBERTA, CANADA T1J 4B1 AND
[2]EASTERN CEREAL AND OILSEED RESEARCH CENTRE, AGRICULTURE AND AGRI-FOOD CANADA, CENTRAL EXPERIMENTAL FARM, OTTAWA, ONTARIO, CANADA K1A 0C6

The accumulation of mono-, disaccharides and fructans, and total carbohydrates varied among winter wheat cultivars possessing different levels of resistance to snow moulds and freezing temperatures. The levels of soluble carbohydrates in crown tissues were influenced by the extent of pre-hardening growth at 20°C and hardening growth in plants at 2°C. The association between carbohydrate accumulation in crowns and snow mould resistance or freezing tolerance were observed only at specific developmental stages. Fructan consisted mostly of oligosaccharides ranging from DP3–DP10 that attained the highest levels in older plants. A subtraction cDNA library yielded an enriched fraction of induced genes related to freezing and snow mould resistance. Most sequences are not homologous to any sequences currently present in GenBank. Preliminary results using an invertase-like probe suggest that the parental lines Norstar and PI181268 differed in their expression of 6-SFT (sucrose:fructan 6-fructosyltransferase) and invertase-like sequences among mRNA populations. Results will be discussed in terms of resistance to freezing temperature and snow mould, and towards the development of winter hardy and snow mould resistant wheat varieties for western Canada.

Key words: winter wheat, fructan, freezing tolerance, snow mould resistance

Introduction

Winter wheat infrequently survives winter stresses in the deep snow regions of central and northern Canadian prairies (Gaudet et al., 1989). A frequent winter stress encountered is exposure to lethal and sub-lethal low temperatures. In many regions, a deep, persistent snow cover insulates the root and crown zone against low temperature injury. Snow cover creates a dark, humid environment with constant soil temperatures between 0°C and –7°C that promotes the development of several different species of psychrophilic fungi that can cause extensive snow mould damage (Gaudet et al., 1989). The prevalence and severity of individual snow moulds is generally governed by the duration of the snow cover and the average temperatures beneath the snow (Bruehl et al., 1966). Therefore, high levels of resistance to low temperatures and snow moulds are a prerequisite in crops adapted to these regions. Among winter cereals, genetic variation exists among cultivars in levels of resistance to snow moulds and freezing temperatures. Snow mould resistance appears to be conditioned by the additive effect of two or three loci (Amano, 1982; Iriki and

Kuwabara, 1993) while freezing tolerance is clearly multigenically controlled (Singh and Laroche, 1988). However, separation of the two phenomena is difficult because both are triggered by a reduction in ambient temperatures (Gaudet and Kozub, 1991).

We are currently studying the effect of plant size and duration of hardening conditions on the accumulation and metabolism of the simple sugars glucose, fructose, sucrose, and complex fructans, and the expression of random cDNA clones, 6-SFT and invertase in winter wheat cultivars differing in resistance to freezing temperatures and snow moulds under controlled environment conditions.

Materials and methods

The snow mould resistant winter wheat (*Triticum aestivum* L. em. Thell) cultivars, 'CI14106', 'PI181268', the freezing resistant and snow mould susceptible cultivar 'Norstar', the moderately freezing tolerant and snow mould tolerant doubled haploid line DH^{++} and the tender and snow mould susceptible line DH^{--} were seeded in a mixture of loam, sand, and peat (3:1:1). Plants were grown in growth chambers with daylength periods for growth and hardening of 18h and 12h, respectively. Light intensities of 350 and 275 $\mu mol \cdot m^{-2}s^{-1}$ of photosynthetic photon flux density, for growth and hardening conditions, respectively, were provided by cool white fluorescent tubes and incandescent bulbs.

Plants were grown (pre-hardening growth) for 2 or 6 weeks at 20°C, and hardened for 0, 1, 3, 4 or 6 weeks at 2°C. Three replicate growth cabinets were used; within each cabinet, combinations of the pre-hardening growth and hardening intervals were arranged in a randomized design. At the different stages of pre-hardening and hardening growth, crown sections (2-cm crown plus stem tissue) were sampled.

For carbohydrate analysis, between 0.5 and 1.0 g of crown tissues were extracted in boiling, distilled water. The total simple sugars glucose, fructose, and sucrose were measured using an enzymatic determination (Boehringer-Mannhiem), UV method at 334 nm. Fructan was determined as the sum of the fructose and glucose contents following 15 min sulphuric acid treatment of the sample hydrolysis (Somani et al., 1987), minus the pre-hydrolysis fructose and glucose contents.

Fructan chromatograms were produced using high-performance anion exchange chromatography (HPAE) combined with pulsed amperometric detection (PAD) in a Dionex® Series 4000 ion chromatograph fitted with a CarboPac® PA-100 column and a PA-100 guard column. A mixture of standards, consisting of glucose, fructose, sucrose, raffinose, 1-ketose, 1,1-ketotetraose and 1,1,1-ketopentose, served as oligosaccharide standards. A fructan extract from *Helianthus tuberosus* served as the polysaccharide standard.

Total RNA was extracted from 1 g of crown tissue using the TRIzol reagent (Life Technologies Inc.). Messenger RNA was obtained using the Hybond-mAP messenger affinity paper (Amersham). Subtraction was done according to Patel and Sive (1996). Cloning, clone selection, slot blot hybridization, northern and southern hybridization and probe labelling techniques were done using standard methods (Ausabel et al., 1997). A sequence of inserts was obtained using the ABA PRISM™ Dye Terminator Cycle Sequencing Reaction Ready Kit (Perkin Elmer) and reactions were analysed using the ABI 373A automated sequencer. Search for sequence homology in GenBank was carried out using BLASTN and BLASTX according to Altschul et al. (1990) for nucleotide data and deduced amino acids.

Results and discussion

Carbohydrate analysis

Low levels of simple sugars were recorded in unhardened crowns and levels rose to maximum levels, in most cultivars to 50–100 mg/g DW and 50–150 mg/g DW, in the 2 and 6 weeks pre-hardening growth treatments, respectively, followed by 1 week hardening. Upon additional exposure to hardening conditions (3 or 6 weeks hardening), simple sugar levels remained stable or decreased slightly. PI181268 attained the maximum level of simple sugars in 6 weeks pre-hardening growth treatment followed by 3 weeks hardening treatment. The association between simple sugars and resistance to either snow moulds or freezing temperatures, although generally weak, was highest in 2 weeks pre-hardening growth, 3 and 6 weeks hardening treatments. Snow mould resistance was primarily associated with high fructose levels, whereas freezing resistance was associated with glucose levels.

Fructan, undetectable in unhardened crowns, increased to near maximum levels following 3 weeks exposure to hardening conditions. In general, the crowns in treatments exposed to 6 weeks pre-hardening growth and extended hardening conditions developed fructan levels approximately double (225–450 mg/g DW) those in the 2 weeks pre-hardening growth treatment (80–320 mg/g DW). The snow mould resistant cultivar CI14106 attained the highest overall levels of fructan in the 2 weeks pre-hardening growth treatment but was equivalent to Norstar and the dihaploid line DH^{++} in the 6 week pre-hardening growth treatment. Approximately 75–80% of all soluble carbohydrates in winter wheat crowns was converted to oligosaccharide, following exposure of plants to 3 to 6 weeks hardening conditions. Younger plants increased fructan production more rapidly in response to hardening conditions than did older plants.

Fructan accumulation was most closely associated with resistance to snow moulds in both the 2 and 6 weeks pre-hardening growth treatments followed by 6 weeks hardening. Fructan and simple sugar accumulation in the snow mould resistant cultivar PI181268 was markedly different than in CI14106, suggesting that more than one form of snow mould resistance may exist.

The greatest proportion of fructan in hardened winter wheat occurred as fructo-oligosaccharides. Peaks representing trisaccharides and oligosaccharides in the range of DP4-DP7 were evident following exposure to hardening conditions for only one week. CI14106 accumulated oligosaccharides more rapidly, and at higher degrees of polymerization compared to Norstar. The existence of more than one peak for each degree of polymerization indicated more than one isomer of oligosaccharide was present.

Identification of DNA sequences induced at hardening temperatures

Subtractive hybridization was useful for enriching sequences related to resistance to snow moulds or freezing temperatures that are induced, or constitutively expressed but upregulated following exposure to hardening conditions. The majority of the 72 randomly sampled clones were specific to the DH^{++} or overexpressed in the DH^{++} line compared to the DH^{--} line. The insert size of the randomly picked clones from the subtracted fraction A_7 ranged from 100 to 320 bp which corresponded to the size range of the A_7 fraction. It was also possible to determine whether clones originated from the Norstar or PI181268 parent.

Four of the 15 randomly selected clones sequenced exhibited a strong homology in nucleotide and deduced amino acid sequence to NAD^+ isocitrate dehydrogenase (IDH) subunits 1 and 2 in *Arabidopsis thaliana*. The high proportion of IDH clones isolated (27%) appeared to be a ligation artifact. None of the other clones shared sequence homology to previously reported low temperature related sequences. Northern analysis of IDH clones showed that this sequence is upregulated at hardening temperatures and more prominently in the DH^{++} line and the Norstar parent (Fig. 1). Similarly, multiple sequences related to invertase were upregulated in the snow mould resistant lines, PI181268 and DH^{++}, following low temperature treatment compared to the snow mould susceptible Norstar and DH^{--} lines.

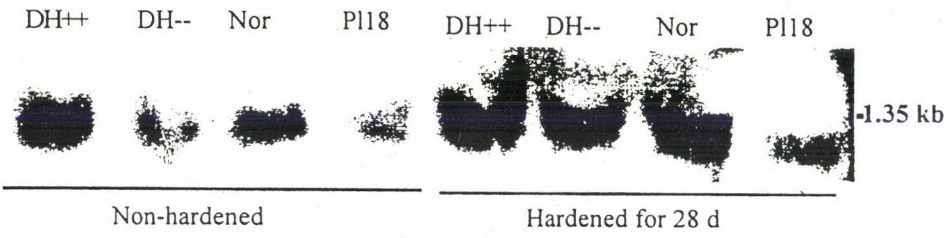

Fig. 1. Northern hybridization of total RNA (30 μg) isolated from the double haploid lines DH^{++} and DH^{--} and parental lines grown for 14 days at control temperature or cold-hardened for 28 days at 2°C. The IDH probe hybridised to transcripts of 1.35 kb

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SELECTION FOR FROST TOLERANCE IN ANTHER CULTURE-DERIVED EMBRYOS AND REGENERATION OF FROST-TOLERANT FERTILE DH PLANTS IN WINTER WHEAT

G. KOVÁCS and B. BARNABÁS

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462, MARTONVÁSÁR, P.O. BOX 19, HUNGARY

The use of anther culture to screen for frost tolerance and to select mutants at haploid level is still limited in cereals. However, the *in vitro* haploid and dihaploid induction of wheat may provide a useful tool for the selection of frost resistance, as the existing or induced tolerance in the microspore or haploid embryo population can easily be screened and fixed in homozygotic form. The possibility of developing frost resistant lines by exposing somatic tissue cultures to subzero temperatures after hardening had been already reported by several research groups, but up till now no data on the use of haploid cultures is available.

In the present experiment the possibility of selecting frost-tolerant haploid embryoids and the regeneration of fertile DH lines from these selected embryos was studied. In the course of the experiment anthers from the F_1 generation of Mv15 (frost-tolerant) × GK Kincső (sensitive) reciprocal hybrids and their parents were cultured on a solidified W14 induction medium. After 5 weeks of induction in the dark the haploid embryos were harvested and cold-treated under controlled phytotronic conditions. The hardened embryos were frozen at −15°C. After freezing, regeneration was carried out using 190-2 regeneration medium and the regenerants were grown till maturity in phytotronic growth chambers. The fertile regenerants were self-pollinated. The progeny generation was tested using the usual frost resistance testing method and the results of the freezing test suggested that about 80% of the DH lines obtained were frost tolerant. According to the data obtained in the experiment, it can concluded that, using the haploid selection method, there is a strong possibility of producing frost-tolerant DH lines from crosses between frost-resistant and sensitive genotypes in a relatively short time. Some of the DH lines regenerated from the frozen embryos of the sensitive parent showed relatively high viability in the freezing test. These results suggest the possibility of selecting for frost-tolerant mutants from a haploid culture.

Key words: anther culture, frost tolerance, haploid selection, wheat

Introduction

Freezing temperature is one of the most severe abiotic stress factors limiting wheat plant growth, productivity and distribution in several countries, especially in continental climates. Low-temperature hardening allows winter wheat to protect critical cell structures and physiological processes during periods of freezing temperature. Genotypes differ in their ability to make physiological and biochemical adjustments that help them to survive low-temperature stress and these differences have been exploited by plant breeders

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to produce winter-hardy cultivars. The exploitable genetic variability for frost resistance has been exhausted within the gene pools of wheat and most evidence indicates that only a small gain can be expected through conventional breeding programmes. Conventional selection methods are highly time-consuming and are not accurate enough to allow for the identification and selection of the small differences that are important in plant breeding (Fowler and Limin, 1997). Recent advances in biotechnology have provided opportunities for wheat breeders to expand their attack on the winter-hardiness barrier. One of the most promising fields within this research is the use of the haploid tissue culture technology. The application of doubled haploids in breeding programmes is now established for a number of crops including wheat (Bedő et al., 1993) and many cultivars have been released from programmes using haploid procedures (Kasha et al., 1995). In the area of molecular genetics, haploid procedures have proved to be very beneficial in developing molecular maps and in analysing quantitative trait loci (QTL). The use of haploid techniques has also been the target of research in establishing useful *in vitro* selection systems in crops, but limited success is reported (Orr et al., 1990).

Breeding for frost resistance in wheat depends not only on the genetic resources available, but to a large extent on the efficiency of the breeding and selection techniques. Recent progress in doubled haploid production techniques for wheat is already sufficient to provide new opportunities for breeding and genetic studies on several agronomically important characters including frost tolerance. Dihaploid plants produced from anther or isolated microspore cultures are homozygous, a condition difficult to obtain by any other breeding method (Zhou et al., 1993). The production of "instantly homozygous" lines can accelerate the development of frost-tolerant varieties and provide "unique" material for genetic and biochemical studies on hardening and frost tolerance mechanisms. Anther- and microspore-derived embryos or calli can also be exploited for *in vitro* tests of frost-tolerance related molecular and genetic studies.

In the literature no data is as yet available on the application of *in vitro* haploid cultures for the study of the mechanisms of hardening and frost tolerance. However, the physiology and genetics of frost tolerance (Chen and Gusta, 1986; Tanino et al., 1990; Galiba et al., 1993) and the possibility of developing frost-tolerant lines by exposing somatic calli to subzero temperatures after hardening have been reported by several research groups (Galiba, 1994; Kovács, 1990). The results of these experiments suggest that besides selecting for already existing frost tolerance it is also possible to induce somaclonal variation for this trait (Lazar et al., 1988). Unfortunately, in several cases the increased frost tolerance induced proved to be the result of epigenetic changes and was no longer detectable in the R_4 generation.

The main aim of our recent work in the field of frost tolerance studies is to establish an effective haploid selection system for producing frost-tolerant, homozygous DH plants from early generations of sensitive × tolerant wheat genotype crosses and to provide breeders with an effective method to speed up their selection procedure.

Materials and methods

Plant material

In the present experiment, two common winter wheat varieties, the frost-tolerant Mv 15 and the frost-sensitive GK Kincső, were studied together with their reciprocal F_1 hybrids. The anther donor plants were raised in the field under optimal conditions. Spikes were collected prior to emergence from the leaf sheath when the microspores were judged to be at the mid- or late-uninucleate stage of development. Around 300 spikes per variety and hybrid combination were collected and cultured to guarantee the representativeness of the existing genetic variation.

Anther culture

Before excision of the anthers, the spikes were surface sterilized with 0.1% $HgCl_2$ solution for 8 minutes and rinsed three times with sterile distilled water. One or two anthers from each spike were examined microscopically to verify the microspore develepmental stage before culturing. Spikes which contained only mid- and late-uninucleate microspores were used for culture. Subsequently, the anthers were excised and inoculated onto Petri dishes (10 × 1.5 cm) containing about 30 ml modified solid W14 (Ouyang et al., 1989) induction medium. The basal W14 medium was supplemented with 2 mg/l 2,4-D, 0.5 mg/l kinetin and 9% maltose at pH 5.5. The number of anthers cultured per spike varied according to the number of florets per spike and the viable anthers per floret; however, each Petri dish contained approximately 100 anthers. The cultures were incubated in the dark at 29°C for 35 days. The number of responding anthers and of induced haploid structures (embryos or calli) were recorded after 35 days of culture. About half the harvested anthers were transferred to a BM medium (Chu et al., 1990) supplemented according to Kovács et al. (1995) with 1 mg/l 2,4-D, 0.5 mg/l kinetin and 5% sucrose for subculturing. This part of the harvested embryos (around 50 embryoids/dish) were subcultured in a growth chamber with 16 hours illumination (45 $mM/s/m^2$ light intensity) at a constant temperature of 27°C for a one-week adaptation period. The remaining embryos were directly transferred onto regeneration medium (Zhuang and Jia, 1983). Plant regeneration were carried out using 190-2 regeneration media (He and Ouyang, 1985) under 16 hours illumination (50 $mM/s/m^2$ light intensity) at a constant 27°C. The young green plantlets obtained were transferred onto 1/2 MS (Murashige and Skoog, 1962) containing only half of the inorganic salt of the MS medium and after two weeks culture they were transferred to soil. The non-hardened regenerants were vernalized for 7 weeks at a constant 2°C. The regenerants were grown in E-15 growth chambers using the T3 climatic programme (Tischner et al., 1997) till maturity. At the end of the programme the percentage of fertile plants and their seed setting were recorded.

Hardening and freezing in vitro

After one week of adaptation the cultures were covered with aluminium foil and hardened using the modified FDA climatic programme in a PGV-36 (CONVIRON) climatic chamber (Tischner et al., 1997). The 6-week FDA programme was preceeded by a one-week adaptation period with a slightly higher temperature (15–17°C) so that mature haploid embryos would be available for freezing. At the end of the hardening period the cultures were frozen with the usual 5-day freezing programme in which the minimum temperature is –15°C for 26 hours in the dark. After freezing, the cultures were maintained at a constant 10°C for 5 days before starting the regeneration (recovery) period.

Freezing test on the progeny generation

The seeds of regenerants from both selected and non-selected populations were germinated for two days in Petri dishes at room temperature, then sown in wooden boxes filled with soil and sand mixture (4:1) and hardened using the FDA climatic programme in PGV-36 climatic chambers (CONVIRON). During the hardening period the plants were given less nutrient solution and water, and during freezing they were not watered at all. The plants were frozen using the above described freezing protocol. After freezing, the leaves were cut off a few centimetres above the soil so that regrowth could be more accurately evaluated and to avoid the risk of infection by fungal diseases. The survival rate was recorded after 18 days.

Results

The results of anther induction (Table 1) suggest that both the parents had a certain androgenic capacity, giving enough embryoids to carry out the selection experiments. According to the data there is no significant difference in anther response between the two genotypes and their hybrids and no reciprocal differences were found. After callus induction Mv 15 gave a significantly lower embryo yield than GK Kincső and the reciprocal hybrids, and a slight but not significant heterotic effect could be detected. Among the two reciprocal hybrids no significant maternal effect could be detected either in anther response or callus induction.

Table 1
Androgenic response of frost-sensitive and frost-tolerant genotypes and their reciprocal hybrids in anther culture

Genotypes and hybrids	No. of anthers cultured	Anther response (%)	Callus induction (%)
GK Kincső	2268	10.7	35.1
Mv 15	3588	8.1 ns	13.3*
GK Kincső × Mv 15	3468	11.0 ns	43.1 ns
Mv 15 × GK Kincső	3288	12.9 ns	36.2 ns

*- Significantly different from the response of GK Kincső at the p = 0.05 probability level; ns - non-significant

The data obtained in the present experiment prove that the process of hardening also takes place at the haploid level, so frost resistance can be tested in a population of haploid embryoids originated from anther culture. After freezing several calli survived the subzero temperature treatment, showing an intensive growth on the regeneration medium (data not shown), but only a few of them were able to regenerate into plantlets. In the case of the sensitive variety GK Kincső about 25% of the haploid calli grew on the regeneration media, showing that even the sensitive calli could survive the subzero temperature treatment after hardening. About 86% of the calli and embryoids of the frost-tolerant variety Mv 15 survived frost treatment, suggesting that differences in frost tolerance can easily be detected in haploid tissue cultures. A slightly different picture can be found in the case of plant regeneration (Table 2). The freezing of embryoids at –15°C greatly decreased the regeneration capacity of all the genotypes and combinations tested, but interestingly enough this treatment significantly increased the green plant regeneration frequency in all cases. The plant regeneration frequency dropped to approximately one tenth compared to the control (non-treated) regeneration experiment in both varieties tested. This drop was not so great in the reciprocal hybrid combinations, showing that heterotic origin itself can provide higher adaptability for the haploid calli. The green plant regeneration frequency was significantly higher in all genotypes and combinations tested, and in some cases only green plants were regenerated. No significant differences were found between the reciprocal crosses, except in the case of green plant regeneration after freezing. It is interesting to note that embryoids derived from the Mv 15 × GK Kincső hybrid regenerated fewer green plantlets after freezing, although the maternal genotype Mv 15 had higher green plant regeneration capacity than GK Kincső.

Table 2

Plant regeneration frequency of haploid embryoids after freezing at –15°C

Genotype	Treatment	Plant regeneration (%)	% of green plants
GK Kincső	control	11.3	25.8
	frozen	1.7***	85.7***
Mv 15	control	25.5	82.6
	frozen	2.1***	100.0**
GK Kincső × Mv 15	control	26.7	50.5
	frozen	5.0***	100.0***
Mv 15 × GK Kincső	control	29.4	33.3
	frozen	7.4***	56.4**

, *, significantly different from the non-treated control at the p = 0.01 and 0.001 probability levels, respectively

According to the data obtained only a few fertile plants could be regenerated after freezing (Table 3). The two parent genotypes gave the lowest fertile plant regeneration, while the two hybrid populations showed approximately 5–8 times higher fertile plant regeneration capacity after freezing. There was no significant difference between the reciprocal crosses in this respect either. The number of fertile plants in this experiments also reflects spontaneous genome doubling, as no chromosome douling agents were applied. After freezing, all the green regenerants were fertile, while in the control regeneration experiment the average rate of fertile plants was only about 40% (data not presented), showing that spontaneous genome doubling could took place during the hardening period.

The average seed set of the regenerants differed greatly even in the case of lines originating from the same treatment, but on average hybrid-derived regenerants gave the highest seed set (Table 3). The frost-tolerant Mv 15 DH regenerants showed the lowest fertility.

Table 3
Number of selected fertile DH plants and their average seed setting ability*

Genotype	No. of fertile plants	Seed set %
GK Kincső	6	30.1
Mv 15	6	19.8
GK Kincső × Mv 15	52	47.9
Mv 15 × GK Kincső	30	34.7

* no statistical analysis was carried out

Seedlings of the progeny generation were tested using the conventional frost resistance testing method used in the Martonvásár phytotron and the survival rate was measured for each DH line in comparison with the control variety (Table 4). *In vitro* selection for frost resistance via the hardening and freezing of anther culture-derived haploid embryos could be a highly efficient method for the production of frost-tolerant DH lines, as the data of the frost resistance test of the seedlings resulted in a significant improvement in frost tolerance in most of the DH lines studied.

Table 4
Survival rate of seedlings of the progeny generation after freezing at –15 C
in the conventional frost resistance test

Genotype	Origin	Survival rate %
GK Kincső	control variety	0.0
	anther culture-derived	0.0 ns
	in vitro selected	4.5 ns
Mv 15	control variety	80.5
	anther culture-derived	85.6 ns
	in vitro selected	88.9 ns
GK Kincső × Mv 15	anther culture-derived	6.9
	in vitro selected	39.7***
Mv 15 × GK Kincső	anther culture-derived	37.2
	in vitro selected	61.9***

***Significantly different from the control variety at $p = 0.001$ probability level, ns - non significant

As was expected, seedlings of the sensitive variety did not survive the strong frost stress applied in this experiment, and the same results were obtained in the case of its DH lines when regenerated from a non-hardened, frozen control experiment. Interestingly enough one DH line obtained from the selection experiment showed as high as 86% survival, suggesting that tolerant mutants might be obtained at low frequency. A high percentage of variously derived seedlings of the frost-tolerant Mv 15 survived the subzero temperature and no significant differences were detected between the various origins. In the case of the reciprocal crosses selection at haploid level seems to be very effective, because in all cases hardened, frozen haploid embryo-derived DH lines tolerated the applied frost stress significantly better than the control, non-selected DH lines. According to our data a significant reciprocal effect could be found between the reciprocal crosses.

Discussion

In the present experiment the possibility of selection for frost tolerance at the haploid level was studied. According to the data presented, it can be concluded that the frost tolerance demonstrable at plant level can also be analysed in haploid tissue cultures. The results of earlier experiments prove that the process of hardening takes place in the case of both individual somatic cell cultures and calli (Steponkus, 1972; Chen and Gusta, 1986), but in the literature no data has been available until now on the application of *in vitro* haploid cultures to study the mechanisms of hardening and frost tolerance. The present data suggest that the use of embryoids and calli originating from anther culture could be a very good tool in such studies, since at haploid level individual gene effects can be studied without the problems caused by the masking effects of different allelic interactions. On the basis of the present experiments it can be assumed that the use of *in vitro* haploid cultures will greatly facilitate biochemical and genetic studies of frost resistance, while parallel examinations at plant and somatic calli level may disclose the effects of ploidy and organization.

The examination of haploid cultures and DH lines of the frost-sensitive genotype GK Kincső offers wider possibilities. The fact that some of its haploid cell lines grow intensively after the strong frost stress applied, and that one frost-tolerant DH line was regenerated suggests the possibility to develop stress tolerant mutant selection methods. Unfortunately the data presented are unsufficient to be sure that the increased frost tolerance originated from real genetic changes (mutation at the haploid level), because in most of the cases improved tolerance may be simply the result of some epigenetic change. Even if the early progeny generations carry the changed phenotype there is no guarantee that the improved character will be steadily transmitted. Further research is required to answer these questions.

It could be cuncluded from the fertility data that the low temperature treatment applied during hardening was the main cause of the high frequency of spontaneous chromosome doubling, as low temperature treatment was used for a long time in traditional experiments to induce polyploidy (Sybenga, 1972).

The data form the frost resistance testing of the DH lines obtained after freezing suggests that the haploid selection system is an efficient way of producing homozygous frost-tolerant DH lines. According to the data obtained it can concluded that selection at the haploid level results in homozygous DH lines with improved frost tolerance in all cases of hybrid combinations, and that the methodology applied in the experiments could be incorporated into wheat breeding programmes, where frost tolerance is one of the target characters.

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IMPROVEMENT OF FROST TOLERANCE IN WINTER WHEAT BY *IN VITRO* SELECTION OF PROLINE-OVERPRODUCING MUTANTS

K. DÖRFFLING[1], H. DÖRFFLING[1], G. LESSELICH[1], E. LUCK[1] and G. MELZ[2]

[1]INSTITUTE OF GENERAL BOTANY, UNIVERSITY OF HAMBURG, D-22609 HAMBURG, GERMANY
[2]PFLANZENZÜCHTUNG GÜLZOW GMBH, RINGSSTRASSE 10, D-18276 GÜLZOW, GERMANY

In several genotypes of winter wheat a significant positive correlation between cold-induced frost tolerance and the accumulation of free proline in the leaves was found in earlier studies. Based on this observation the hypothesis was developed that proline accumulation and frost tolerance are causally connected. To test this hypothesis proline overproducing mutants from a Finnish winter wheat variety (Jo 3063) were produced by the *in vitro* technique using embryogenic calli which were exposed to hydroxyproline (Hyp) as selection agent. Regenerated hydroxyproline-resistant plants as well as their F_1 to F_5 progenies proved to possess higher proline content in their leaves as well as higher frost tolerance in leaves and whole plants after cold hardening under growth chamber and field conditions. The mutation seems to be due to a single incompletely dominant gene. Besides providing substantial evidence for a role of proline in the process of frost tolerance development the study opens the possibility of improving winter hardiness by this biotechnological method.

Key words: frost tolerance, hydroxyproline (Hyp) resistance, *in vitro* selection, proline, *Triticum aestivum*

Introduction

Studies on several plant species and their various varieties including winter wheat and winter barley which develop frost tolerance during the process of cold hardening have shown that free proline accumulates in leaves and shoots to considerable amounts during this phase, and that the proline accumulation is positively correlated to genotype-specific frost tolerance (van Swaaij et al., 1987; Dobslav and Bielka, 1988; Dörffling et al., 1990). Proline is thought to play a protective role in plants subjected to several stresses including frost (Delauney and Verma, 1993). In order to provide evidence for proline as being one of several factors determining the degree of frost tolerance, we selected lines of winter wheat with an increased accumulation of proline by the *in vitro* technique. These lines and their progenies proved to possess improved frost tolerance as well (Dörffling et al., 1993; 1997). The most recent results of these studies, especially simulated field studies with F_5 progenies are described in this paper.

Materials and methods

Embryogenic calli of a Finnish winter wheat, cv. Jo 3063, were exposed to 20 mM Hyp for 12 weeks. Controls were grown on Hyp-free Gamborg B5 medium. The Hyp-resistant calli and controls were regenerated, and the regenerated plants were transferred to soil and cold-hardened at 2°C for several weeks. The procedure has been described in detail in previous papers (Dörffling et al., 1993; 1997). The proline content of the fully grown leaves was measured according to Bates et al. (1973). Freeze-dried material (10–20 mg DW) was boiled for 45 min in 3 ml pure water, and 1 ml was used for the ninhydrin reaction. The frost tolerance of the leaves was determined by means of the conductivity method after 4 weeks hardening at 2°C. The frost tolerance of whole plants was also determined. Plants cold-hardened in a growth chamber at 2°C were exposed to frost treatment down to –20°C in steps of 2°C/h. Survival rates were calculated after further cultivation for four weeks at 2°C and finally six weeks at about 18°C in a greenhouse. Field-grown potted plants were exposed in the first days of January to an artificial freezing test at –25°C for 1, 3, 6 and 22 hours and then exposed again to natural field conditions. Survival rates were calculated at the end of April.

Results and discussion

From 6018 embryogenic calli exposed to 20 mM Hyp only a few survived and nine regenerated. The mean proline content and mean frost tolerance of the cold-hardened regenerated plants (R_0) was significantly higher as compared to regenerated cold-hardened *in vitro* controls (Dörffling et al., 1993). Because of self-sterility the regenerated Hyp-resistant plants and the *in vitro* controls were pollinated with pollen from the wild type cv. Jo 3063. Back-crossed Hyp offsprings of the R_0 generation also showed higher proline contents and higher frost tolerance in comparison to the wild type and the progenies of the *in vitro* controls. The F_1 Hyp plants and the *in vitro* controls were self-pollinated and the seedlings obtained from three R_0 Hyp-resistant plants were again investigated after cold hardening at 2°C for four weeks. The F_2 generation showed a segregation in a 3 to 1 phenotypic ratio (less tolerant : more tolerant) with regard to frost tolerance and proline contents (Dörffling et al., 1997).

In the F_3 generation homozygous lines with significantly increased frost tolerance (lower LT_{50} values) and increased proline contents in comparison to the wild type were obtained. From nine investigated Hyp-resistant progenies six lines had significantly lower mean LT_{50} values (down to –14.8°C) than the wild type (–12.1°C) and all had significantly lower LT_{50} values than the *in vitro* controls (–10.6°C). The same was observed with regard to proline accumulation: six of the nine investigated Hyp lines showed significantly higher mean proline contents (up to 8.8 mg/g DW) than the wild type (4.7 mg/g DW) and all nine lines had higher values than the *in vitro* controls (2.7 mg/g DW). The traits "increased frost tolerance" and "increased proline content" were significantly correlated.

Whole plant assays with F_4 and F_5 progenies cultivated under growth chamber and field conditions confirmed the improved frost tolerance, which was connected with increased proline accumulation. The F_4 generation of one Hyp-resistant mutant was cultivated and tested under controlled conditions in a freezing test with temperatures down to –20°C, each step lasting 1 hour. In the range between –14°C and –18°C the mutant showed up to 33 % better survival than the wild type and the *in vitro* controls.

In the F_5 generation seedlings of five Hyp-resistant progenies and the wild type were cultivated under field conditions and exposed at the beginning of January for 1, 3, 6 and 22 hours to an artificial frost of –25°C in a freezing chamber. Survival rates were calculated at the end of April (Fig. 1). After exposure for 3 hours at –25°C the Hyp-resistant mutants had up to 2.6 times and after 6 hours at –25°C up to 4 times higher survival rates than the wild type. After 22 hours at –25°C no plant of the wild type, but up to 14% of the mutants had survived.

For proline measurement Hyp mutants (F_5 generation) and wild type plants were cultivated under controlled conditions and hardened for 2 weeks at 2°C. The proline accumulation in the mutants was found to be much higher than in the wild type (Fig. 2).

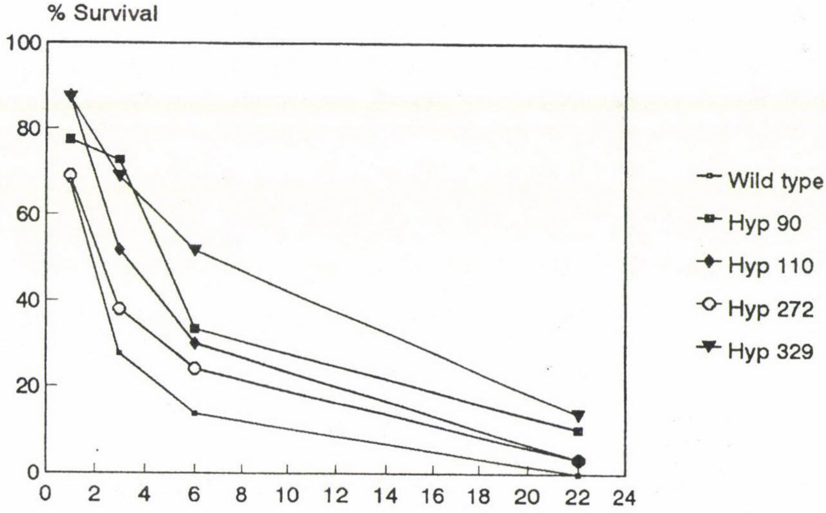

Fig. 1. Effect of frost treatment (up to 22h at –25°C) on the percentage survival of four Hyp mutants (F_5 generation) in comparison to the wild type (Jo 3063). All plants were grown before and after the (artificial) frost treatment under field conditions. Each point is the mean of two experiments with 30 plants each

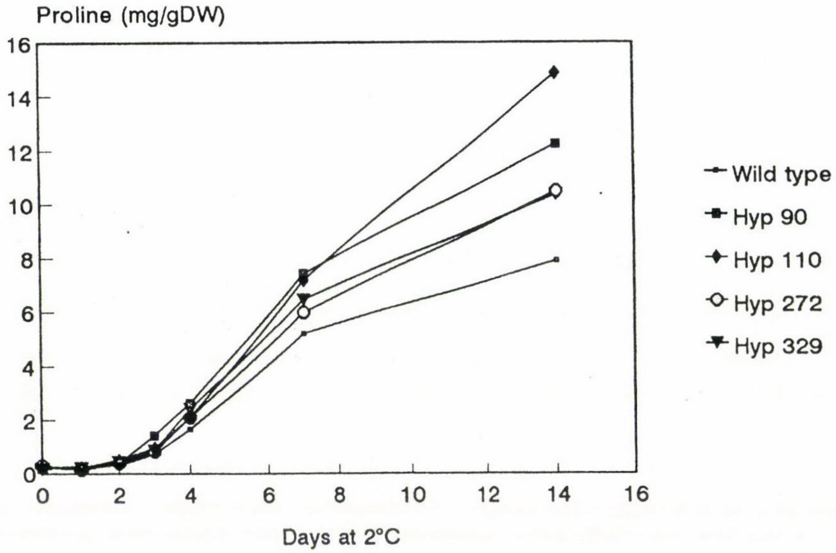

Fig. 2. Proline accumulation in secondary leaves during cold hardening at 2°C of four Hyp-mutants (F_5 generation) in comparison to the wild type (Jo 3063). Each point is the mean of at least 12 plants

These results confirm our earlier findings (Dörffling et al., 1993; 1997; Tantau et al., 1991) that a close relationship exists between proline accumulation during cold hardening and frost tolerance in winter wheat. The hardened Hyp-resistant mutants possessed higher proline levels as well as higher frost tolerance in comparison to the wild type. The fact that this correlation was found here in the F_5 progeny, as well as before in the F_1 to F_4 progenies is further proof that the *in vitro* selected trait 'increased proline accumulation', which seems to be connected with the trait 'increased frost tolerance', is genetically stable. Work is now in progress to study the efficiency of these traits under real field conditions.

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BREEDING FOR WINTER HARDINESS IN CEREALS

D. B. FOWLER and A. E. LIMIN

CROP DEVELOPMENT CENTRE, UNIVERSITY OF SASKATCHEWAN
SASKATOON SASKATCHEWAN, CANADA S7N 5A8

The growth of all commercial crop species is limited by low-temperature (LT) stress at some stage in their life cycle. Plants differ in their ability to make physiological and biochemical adjustments that help them to survive LT stress and these differences have been exploited by plant breeders to produce winter hardy cultivars. However, while much is known about plant LT response, the maximum cold-hardiness potential within winter cereal species has reached a stubborn plateau that has not been raised for decades.

Advances in biotechnology have provided opportunities for plant breeders to expand their attack on the winter-hardiness barrier that has frustrated them for so long. Exploitation of this new technology to produce adapted, super-hardy cultivars will require close cooperation between plant breeders and biotechnologists. This interdisciplinary effort will be expensive and immediate breakthroughs should not be expected, but progress to date suggests that we now have the tools to identify the pieces of the winter-hardiness puzzle.

Key words: winter cereals, cold hardiness, selection methods, gene transfer, gene regulation, gene expression

Introduction

Winter annual crops overwinter as seedlings that must survive numerous winter stresses. Among these many stresses, low-temperature (LT) injury is the main cause of winterkill.

In order to cope with LT stress, plants have evolved protective mechanisms that are temperature regulated and involve cold acclimation processes which can be stopped, reversed and restarted. Because cold acclimation is an active process that is accompanied by growth at low temperatures, these protective mechanisms also provide winter annuals with a competitive advantage over summer annuals by lengthening the effective growing season and positioning the plant to capitalize on favourable weather conditions. The cold-responsive genetic system responsible for LT adaptation and its regulation and interaction with the environment have presented plant breeders responsible for winter cereal cultivar improvement with many complex challenges.

Genetic differences in the ability to make morphological, physiological and biochemical adjustments that help plants to survive LT stresses were exploited by early farmers and modern plant breeders when lines with exceptional winter survival under field conditions were selected. However, there has been a notable absence of improvement in the maximum cold hardiness potential of cereals in this century (Grafius, 1981; Fowler and Gusta,

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1979). In fact, modern agronomic practices have permitted the cold hardiness potential of new cultivar releases to decrease with time in most traditional production areas. This means that there is still considerable exploitable genetic variability for winter hardiness available for use in cultivar improvement within most established production areas of the world (Gilliland and Fowler, 1988). In contrast, all the efforts of modern science have been unable to produce the super-hardy cultivars needed to expand winter crop production into regions requiring a level of cultivar LT tolerance superior to that found in the land races selected by early farmers.

This paper will focus on our efforts to integrate recent advances in science into the University of Saskatchewan winter wheat breeding programme. This programme has had a mandate to expand the traditional winter wheat production area of the North American Great Plains into the spring crop production region of western Canada. Western Canada, which contains 85% of the arable land in Canada, and Siberia have the coldest climates for crop production of any large agricultural region in the world.

The scientific literature on LT tolerance in plants is voluminous, including a host of books, chapters, conference proceedings and scientific papers. However, space limitations have restricted most of our discussion to pertinent papers that have been published by our programme. This published work includes two recent reviews (Limin and Fowler, 1991; Fowler et al., 1993) and a large number of scientific publications, each of which contains a long list of references to related work published by other groups.

Selection methods

A large number of genes with small effects and complex interactions determine the phenotypic expression of LT tolerance. Current methodology for measuring LT tolerance gives poor resolution of small phenotypic differences and most measurements lack the precision necessary for efficient single plant selection. Many procedures are also destructive, which complicates their use for selection in plant breeding programmes.

Exposure of plants to low temperatures produces myriad changes in morphological, biochemical and physiological characters that can be measured on the plant. Phenotypic differences in these characters are often highly correlated with plant freezing tolerance and these relationships have been exploited in cold hardiness selection programmes. For example, plant erectness in winter cereals, tissue water content and cell size can provide simple, rapid, non-destructive measures of cold-hardiness potential on a single plant (Fowler et al., 1981; Brule-Babel and Fowler, 1989; Limin and Fowler, 1994). However, high experimental errors limit the usefulness of these cold-hardiness indicators to selection for large differences among plants in preliminary screening programmes.

Controlled-freeze tests on plants that have been cold acclimated in controlled environments are routinely used to measure LT tolerance in physiological and genetic studies. Difficulty in reproducing cold acclimation rates in repeat experiments severely limits the resolution of controlled-freeze tests that employ a single minimum temperature and this procedure has been responsible for misleading conclusions in genetic studies (Fowler et al., 1993). In contrast, LT_{50} estimates, which are determined from a series of test temperatures, have provided the highest precision and heritability of all cold-hardiness prediction tests (Fowler et al., 1981).

The cold tolerance of winter cereal crowns is reduced by prolonged exposure to sub-lethal temperatures and, as a consequence, both temperature and exposure time are important variables in controlled-freeze test procedures. The expected LT_{50} for different exposure times (T) to constant temperature can be calculated from the equation:

$$LT50_{(T)} = LT50_{(0)} + 5.72 + 1.53 * LOG(T)$$

Where T is the number of days that plants are exposed to a constant LT stress. $LT50_{(0)}$ is determined using a series of test temperatures where the LT stress is removed as soon as the crown samples are exposed to a predetermined minimum temperature (Limin and Fowler, 1988). The predictable relationship between time and temperature has meant that survival time at below freezing temperatures can also be used to identify differences in plant LT tolerance (Thomas et al., 1988).

Linked markers can simplify the selection for complex genetic traits in plant breeding programmes. The development of restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplification fragment length polymorphism (AFLP) and microsatellite molecular mapping techniques has provided an opportunity for the production of detailed LT gene linkage maps. So far, mapping efforts have focused on the chromosome regions associated with the homoeoalleles *Vrn1* in wheat (Galiba et al., 1995), *Sp1* in rye (Plaschke et al., 1993) and *Sh2* in barley (Laurie et al., 1995) because these regions appear to have a significant influence on plant cold hardiness. However, with as many as 15 out of 21 chromosomes in wheat having an influence on LT tolerance (Stushnoff et al., 1984), there is still considerable work to be done before the full potential of mapping-assisted selection can be realized in cereal breeding programmes.

Antibodies raised against proteins produced by LT-induced *Triticeae* genes (Houde et al., 1992) have provided an immediate practical opportunity to simplify cold hardiness selection procedures in breeding programmes. LT-induced expression of the *Wcs120* gene family in wheat, as measured by densitometry scanning of Western blots, closely follows changes in LT_{50}, thereby providing a direct means of quantifying phenotypic differences in the LT tolerance of cereals (Fowler et al., 1996a).

In spite of the opportunities offered by cold hardiness indicators, controlled-freeze testing and molecular markers, most of today's winter cereal breeding programmes still rely heavily on field screening as the final measure of plant winter survival potential. Field testing is simple, inexpensive, and does not require access to specialized facilities or cooperating programmes with conflicting priorities. Unfortunately, the opportunity for selection in field trials only occurs once a year, winters that provide critical selection temperatures usually occur infrequently, and it is often necessary to grow trials outside the target production area to obtain adequate selection pressure (Fowler et al., 1993). In addition, non-uniform stress levels due to variable snowcover often result in high experimental errors that reduce within-trial selection efficiency. However, the limitations associated with field trials can be minimized by careful site selection, the strategic use of reference cultivars, and the use of experimental techniques developed to adjust for non-uniform stress levels (Fowler, 1979; Fowler et al., 1981). When properly managed, field trials still provide the simplest, most reliable and repeatable measures of plant cold hardiness potential of any of the selection methods that are currently available to plant breeders.

Within-species selection

Genetic studies indicate that LT tolerance is a highly heritable, complex quantitative trait that is strongly influenced by environment. Complex genetic interactions should not be unexpected considering that the induction of the genetic system regulating LT tolerance is associated with a multitude of biochemical, physiological and morphological changes in the plant (Levitt, 1980). The magnitude of the LT-induced changes also suggests that many genes could have an indirect influence on low-temperature tolerance and/or there is a cascade of changes controlling gene expression during acclimation that involves common genetic pathways with divergent links.

LT tolerance is primarily under nuclear control and the cell cytoplasm normally has little, if any, direct influence on the expression of this character (Limin and Fowler, 1984; Brule-Babel and Fowler, 1988). Analyses of within-species genetic variability has identified significant general and specific combining ability effects with a preponderance of additive variance (Sutka, 1981; Stushnoff et al., 1984) for LT tolerance.

While it is generally assumed that there are a large number of genes with small effects and complex interactions that determine the phenotypic expression of LT tolerance, field studies conducted under the extreme winter conditions in western Canada indicate that the genotype by environment interaction for this character is small relative to the error associated with individual measurements (Fowler et al., 1981). This once again suggests that the main restriction on selection for LT tolerance is poor ability to measure differences in this character.

When field screens have been effective, we have had little difficulty identifying segregates with cold hardiness levels approaching the maximum reported for winter wheat (Gilliland and Fowler, 1988). These results support the notion that there is considerable unexploited genetic variability for LT tolerance available to plant breeders who are developing cultivars for regions with low winter stress. In contrast, the inability of plant breeders to increase maximum cold tolerance levels in this century strongly suggests that all of the available LT genes had been previously concentrated in hardy land races within winter cereal species.

Interspecific and intergeneric gene transfer

The search for superior LT tolerance genes has been expanded to include attempts at interspecific and intergeneric transfers. There are considerable differences in the maximum LT tolerances found in different winter cereals (Fowler et al., 1977; Fowler and Carles, 1979; Limin and Fowler, 1981; 1985) and the possibility that genes can be transferred between species to increase the genetic variability available to winter cereal breeding programmes has been explored. However, these attempts have done little more than demonstrate the difficulties that must be overcome before the full potential of superior species-specific cold-tolerance gene expression can be captured through interspecific gene transfers in plant breeding programmes.

The superior LT tolerance of rye was found to be suppressed when combined in tetraploid (Limin et al., 1985) and hexaploid (Dvorak and Fowler, 1978) wheat backgrounds. Artificially synthesized ABD genome hexaploid wheat (Limin and Fowler, 1982) also demonstrated the non-additivity of closely related genomic systems. Further investigation of LT gene expression in hybrids among *Triticeae* species (Limin and Fowler, 1988; 1989) led to the conclusion that chromosome dosage or ratios influence LT tolerance by shifting competitively balanced systems towards the parent with the greatest chromosome number. Molecular investigations of these hybrids has subsequently revealed that highly conserved and coordinately regulated LT-induced gene families of both species are expressed in interspecific crosses (Limin et al., 1995). However, these genes were not expressed independently and the degree of LT gene expression in these interspecific crosses was regulated at the transcriptional level by the higher ploidy parent.

These observations indicate that, in order to successfully exploit alien genetic variability for LT tolerance, we must first acquire a greater understanding of the complex genetic mechanisms that plants have evolved for the efficient integration of LT responses into the daily processes of survival, growth and reproduction. These investigations have started with a search for the principle mechanisms that winter cereals use to regulate LT gene expression.

Regulation of low-temperature tolerance gene expression

Structural genes can be turned on and off by regulator genes (Jacob and Monod, 1961). These types of integrated regulatory systems allow plants to react to ever-changing environmental conditions and synchronize multifactorial physical, biochemical and morphological responses (Guy, 1990; Thomashow, 1990), all of which are of concern to plant breeders. However, we have had great difficulty in separating the genes responsible for LT acclimation and cold hardiness from those associated with metabolic adjustments to LT.

Intraspecific differences in LT tolerance in winter cereals are controlled by mainly additive gene action. However, at least one gene on chromosome 5A of wheat has a dominant effect for LT tolerance that is normally expressed in association with the recessive *vrn1* allele for winter growth habit (Brule-Babel and Fowler, 1988). This region of chromosome 5A, and homoeologous loci in other cereals, appear to play an especially important role in determining LT tolerance. Evidence that LT tolerance genes may be very tightly linked to the *vrn1* locus in wheat (Galiba et al., 1995) and homoeoallelic loci in barley (Hayes et al., 1993) has been reported. It has also been suggested that vernalization and LT responses are interrelated (Fowler et al., 1996b) and that *vrn1* may be pleiotropic, affecting both growth habit and LT tolerance in wheat (Brule-Babel and Fowler, 1988; Roberts, 1990). The fact that spring growth habit may have evolved through increased gene dosage due to the duplication of winter habit alleles at this locus (Halloran, 1967) provides further opportunity for speculation into the evolutionary and regulatory significance of this gene complex. Given their apparent importance in conditioning LT responses in cereals, it is necessary that priority be given to experiments designed to unravel the molecular mysteries associated with the *vrn1* complex and related developmental regulators.

Molecular analysis has shown that genes located on chromosome 5A have a regulatory effect on the expression of LT-induced genes on group 6 chromosomes of wheat (Limin et al., 1997). Developmental factors associated with the vernalization requirement determined by *vrn3* on chromosome 5D were also associated with increased cold tolerance. Molecular studies designed to investigate these interactions have demonstrated that the regulatory influence exerted by the vernalization genes over LT-induced structural gene expression occurs at the transcriptional level in winter cereals (Fowler et al., 1996a). The level and duration of gene expression determine the degree of LT tolerance and the vernalization genes have been identified as key developmental factors responsible for the duration of expression of LT-induced structural genes (Fowler et al., 1996b). As a consequence, spring habit cultivars cannot maintain LT-induced genes in an up-regulated condition and they are unable to achieve the same levels of cold tolerance as winter habit cultivars.

While the structural genes within the *Triticeae* have a high degree of homology and the regulation of cold tolerance expression is operational across genomes, there are large differences between species in the maximum LT tolerance they can achieve. Among the cereals, rye is the most responsive to LT induction, producing a more rapid rate of cold acclimation and up-regulating LT-associated genes to higher levels than other species (Fowler et al., 1996a; 1996b). However, present genetic evidence indicates that plant breeders will have to wait until we have a better understanding of the genetic cascade controlling gene expression during cold acclimation before they will have access to this superior genetic variability for cultivar improvement in related species.

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INTERRELATIONSHIP BETWEEN FROST TOLERANCE, DROUGHT AND RESISTANCE TO SNOW MOULD (*MICRODOCHIUM NIVALE*)

K. WIŚNIEWSKI[1], B. ZAGDAŃSKA[2] and M. PROŃCZUK[1]

[1] PLANT BREEDING AND ACCLIMATIZATION INSTITUTE, RADZIKÓW, PL. 00-950 WARSAW P.O. BOX 1019, POLAND
[2] WARSAW AGRICULTURAL UNIVERSITY, RAKOWIECKA 26/30, PL. 02-528 WARSAW

It has been shown that the relationship between the frost tolerance of *Lolium perenne* and its tolerance to *Microdochium nivale* is of great importance. The frost resistance of winter wheat is correlated with plant dehydration tolerance. Our results indicate that the more intensive water loss from the seedlings of Mironovskaya 808 is determined by the ability to tolerate higher levels of tissue dehydration. By contrast, in San Pastore, in which the intensity of water loss is low, the high intercellular water content becomes the reason for frost damage to this cultivar.

Key words: *Triticum aestivum*, *Lolium perenne*, *Microdochium nivale*, frost resistance, drought

Introduction

The main critical factors for the successful overwintering of plants under the climatic conditions of Poland are: low temperature, freeze-induced desiccation and infection by pathogenic fungi at low temperature.

It is well known that biotic and abiotic stresses induce the synthesis of the same type of secondary metabolites. The synthesis of phenylpropanoids exemplifies this general response. Phenylalanine gives rise to various metabolites, including lignin and suberin, which are induced by the hypersensitive reaction in plants infected by pathogens, but their deposition also increases resistance to low temperature, probably by acting as a water barrier (Dixon and Paiva, 1995). Other metabolites, such as anthocyanins, are induced both at low temperature (Christie et al., 1994) and in plant tissues infected with pathogens and aflatoxins; the derivatives of flavonoids protected plants against UV irradiation (Lois, 1994).

We have attempted to relate the tolerance to *Microdochium nivale,* a pathogen which develops under snow cover, with the cold tolerance of *Lolium perenne* strains. In addition, tolerance to drought and cold has been measured in winter wheat cultivars. Tolerance to both these stresses depends on tissue dehydration. On the basis of the techniques elaborated an attempt has been made to look into the possible homoeology of signal transduction for biotic and abiotic stresses.

The most injurious fungus in grasses is the pink snow mould *Microdochium nivale* (Fries) Samuel & Hallet, previously known as *Fusarium nivale* (Fr) Ces. *Microdochium nivale* is tolerant to low temperature, and severe plant damage by this pathogen occurs in the temperature range between 0°C and 5°C, but the pathogen cannot survive freezing (Smith, 1980; Sanders and Cole, 1981). On the other hand *Microdochium nivale* is able to infect plants during the active growth stage (Hömmö, 1994). The unsatisfactory efficiency of selection for plants resistant to *Microdochium nivale* and frost under natural conditions resulted in the development of reliable laboratory techniques which allow the estimation of plant resistance to any stress. These laboratory techniques proved to be reliable in the evaluation of frost tolerance and resistance to *Microdochium nivale,* as well as frost and drought resistance, and are helpful in breeding programmes for winter and drought hardiness.

Materials and methods

Evaluation of fungal resistance. Plant material

The experiments were carried out on perennial ryegrass (*Lolium perenne* L.) var. Arka (Polish origin) and var. Gremie (German origin) and on nine breeding strains as well as on the winter wheat (*Triticum aestivum* L.) cultivars Mironovskaya 808 (frost-resistant) and San Pastore (frost-sensitive). Twenty-five seeds (in four replicates) of every cultivar were placed side by side on a wet filter paper strip (22.5 × 5 cm) and covered with the same strip. The strips were rolled and put vertically (embryos downwards) into plastic boxes, after which the seeds imbibed in darkness at 4°C. The boxes were then transferred to a climatic chamber (type KTLK 1250, Germany) and the seedlings were grown for ten days under the following conditions: temperature 18°C during daytime and 14°C at night, photoperiod 18 h, photosynthetic photon flux density (PPFD) of 260 μmol m^{-2} s^{-1} and relative humidity 70% during daytime and 80% at night. The plants were fertilized with Knopp solution supplemented with Hoagland's micronutrients. Plant resistance to snow mould was evaluated by a cold chamber test. Twenty plants per pot at the four-leaf stage in four replications were acclimated in a climatic chamber at 4°C with a 16h photoperiod and PPFD of 120 μmol m^{-2} s^{-1}. After 14 days of hardening the plants were inoculated and covered with moistened blotting paper and plastic foil to keep high humidity. The inoculum was prepared by growing the fungus in soil medium (Prończuk and Prończuk, 1987) at 18–20°C for 7 days in darkness. After colonization by the mycelium the soil medium was macerated. Inoculation was made by adding 1 g of the inoculum per plant. Four of the eight experimental replications were inoculated with *M. nivale*, while the other four replications were not inoculated. The inoculated plants were incubated for 30 days at 1°C in darkness. At the end of the incubation period the blotting paper was removed and the plants were transferred to a growth chamber. After a 10-day regrowth period the percentage of surviving plants was calculated.

Evaluation of frost resistance

The frost resistance of plant breeding materials of winter wheat (*Triticum aestivum* L.) and perennial ryegrass (*Lolium perenne* L.) was evaluated by the method of artificial freezing of the seedlings. Ten-day-old plants were subjected to the following hardening (acclimation) conditions: temperature 2°C, photoperiod 24 h, PPFD of 120 μmol m^{-2} s^{-1} and then an additional two days at – 2°C (ryegrass) or – 4°C (winter wheat) in darkness. The seedlings were frozen in a climatic chamber for 20 h at freezing and thawing rates of 1.5°C per hour. The freezing temperature depended on the level of plant hardiness. After thawing in darkness the seedlings were kept at 2°C for two days with a 24 h photoperiod and reduced PPFD (90 μmol m^{-2} s^{-1}). Regrowth was carried out for two days under the initial conditions. After the regrowth period the percentage of surviving plants was evaluated. Plant survival was estimated in terms of LT_{50} and plotted as a function of temperature.

Evaluation of dehydration tolerance

Dehydration tolerance was measured according to the method of Długokęcka and Kacperska-Palacz (1978), i.e. the critical water saturation deficit (WSD causing 50% of tissue injury) was estimated. The critical water saturation deficit for a given leaf was estimated after desiccation of the detached leaf blade in a vacuum desiccator (2.7×10^3 Pa) over anhydrous $CaCl_2$ for 2, 4 or 6 h. The degree of injury caused by a given desiccation period was plotted against the respective water saturation deficit values and the WSD value causing 50% injury (critical WSD) was interpolated. The water saturation deficit (WSD) in the studied leaves was calculated according to Stocker (1929). Rehydration of the leaves to full turgor was performed in a humidity chamber for 16 h. The growth of the leaves during tissue rehydration was not observed. The degree of injury evoked by cell dehydration caused by tissue desiccation under vacuum was evaluated by measuring the amount of intracellular electrolytes released to the medium (distilled water) as a result of injuries to cell membranes caused by dehydration. The amount of electrolytes released from the tissues was determined by measuring the electric conductivity of the diffusate conductometrically. The desiccation injuries were expressed as an index of tissue injury (I_D) calculated according to the formula:

$$I_D = (L_D - L_0/1 - L_0) \times 100\%$$

where L_D and L_0 are mean electrolyte leakage from dehydrated and non-dehydrated (control) leaf tissues, respectively, expressed as a percentage of the total leaf intracellular electrolyte content.

Evaluation of dehydration avoidance

The ability of the tissues to avoid dehydration was estimated from changes in leaf fresh weight during desiccation. The amount of water loss from 1 g of dry matter per min during a desiccation period which caused 50% tissue injury was taken as a criterion of the ability of the tissues to avoid dehydration.

Results and discussion

The distribution of *Lolium perenne* strains surviving frost and *Microdochium nivale* infection under laboratory conditions illustrated in Fig. 1 shows no relationship between these two plant characters. The correlation coefficient is insignificant (r=0.20). However, the strains marked by circles are worth further investigation. Since a concomitant relation exists between tolerance to *Microdochium nivale* and frost, strain S-2 is resistant both to the pathogen and to frost, while strain R-21 is susceptible to both stresses. It has been shown that individual isoforms of pathogen-related proteins are produced at low temperatures (Hömmö, 1994).

During cold acclimation the state and progress of the frost tolerance level of different cultivars of winter wheat were monitored in terms of LT_{50}. The initial level of frost tolerance of San Pastore, a frost-sensitive cultivar, was similar to the frost tolerance level of Mironovskaya 808, one of the most frost-resistant wheat cultivars (Fig. 2). At the end of the first step of acclimation at 2°C, the LT_{50} temperature of Mironovskaya 808 was −16.5°C, whereas that of San Pastore was −11.5°C. During the second stage of cold acclimation, induced

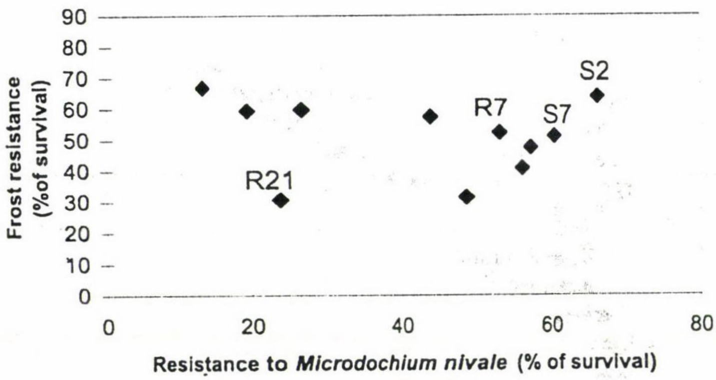

Fig. 1. Relationship between resistance to *Microchodium nivale* and the frost resistance of ryegrass varieties and strains (as a % of plant survival)

by a subfreezing temperature (–4°C) both cultivars developed further frost tolerance and achieved LT_{50} levels of about –22°C for Mironovskaya 808 and about –13°C for San Pastore. It should be underlined that both cultivars are used as standard cultivars during the screening of wheat breeding materials.

Plant drought resistance and frost resistance seem to depend among other factors on a higher stability of the cell membranes under dehydration (Levitt, 1980). Therefore, attempts were made to examine whether the development of plant dehydration resistance was correlated with frost tolerance. Since dehydration resistance, according to Levitt's terminology, is due either to dehydration avoidance and/or dehydration tolerance, both mechanisms were estimated for the frost-resistant Mironovskaya 808 and the frost-susceptible San Pastore during cold acclimation.

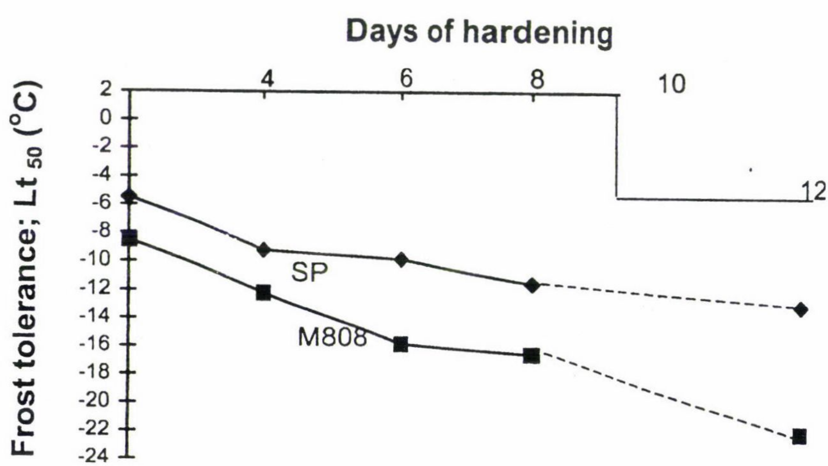

Fig. 2. Frost tolerance changes in winter wheat seedlings of Mironovskaya 808 (frost-resistant cultivar) and San Pastore (frost-sensitive cultivar) during cold acclimation

The data presented in Fig. 3 indicate that the drought resistance of winter wheat leaves is due to the avoidance of water loss from the tissues and also to the tolerance of cell dehydration. The dehydration tolerance of young leaves of both cultivars is high, i.e. the critical water saturation deficit (WSD) of Mironovskaya 808 amounts to 82% and that of San Pastore to 75%. During the acclimation period, the tolerance level is practically constant for Mironovskaya 808 but is reduced to 60% for San Pastore.

The ability of San Pastore to avoid dehydration was found to increase slightly: the rate of water loss under desiccation conditions decreased from 32 mg H_2O per min per g dry matter to about 26 mg H_2O. In the leaves of Mironovskaya 808 the intensity of water loss under desiccation was rather low and slowly increased during cold acclimation. Thus, the results indicate that the more intensive water loss from the seedlings of Mironovskaya 808 was determined by the ability to tolerate a higher level of tissue dehydration. In contrast, in San Pastore, in which the intensity of water loss is low, high intercellular water content becomes the reason for frost damage in this cultivar.

Any attempt to predict winter survival demands the consideration of at least two parameters: cold and disease stresses. Many of the genes responding to biotic and abiotic stresses involve basic metabolisms such as photosynthesis and respiration (Bray, 1997). It is commonly known that genotype-dependent resistance to frost and drought is closely related to the intensity of both these processes (for wheat see Zagdańska et al., 1992). Also, rye cultivars resistant to

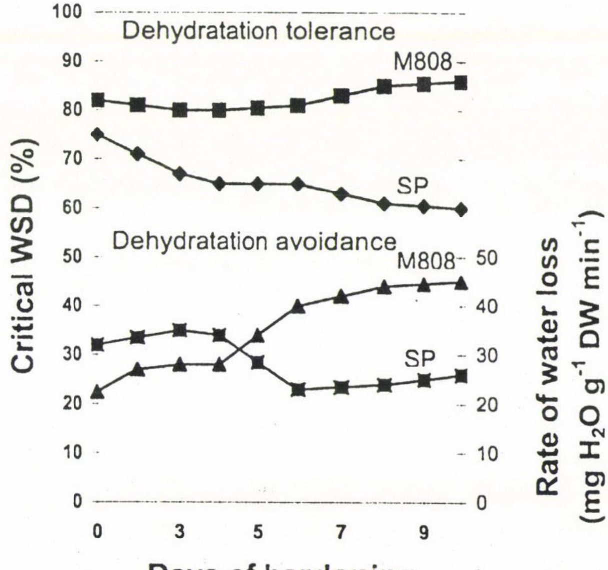

Fig. 3. Changes in dehydration tolerance (critical WSD) and dehydration avoidance (rate of water loss) during the cold acclimation of seedlings of Mironovskaya 808 and San Pastore

Microdochium nivale are characterized by a high intensity of both photosynthesis and respiration (Koczowska and Packa, 1986). Recently more and more reports have appeared on the interlinkage of the regulation of stress-induced genes. According to Hajela et al. (1990) water stress induces cold-regulated genes. The antifreeze proteins in rye have been identified and proved to be glucanase- and chitinase-like proteins which belong to the pathogen-related proteins (Antikainen et al., 1996). Of course, a great deal of work will be required before a selection strategy can be developed for the concerted breeding for cold and pathogen resistance in overwintering plants.

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WINTER HARDINESS OF BREAD WHEATS DERIVED FROM SPRING × WINTER CROSSES

H.-J. BRAUN

CIMMYT, P. K. 39 EMEK, 06511, ANKARA, TURKEY

A survey among winter wheat breeding programmes showed that the cold, or "winter" hardiness of Bezostaya 1 was sufficient for the target area of 61 of the 71 responding programmes. Thus, a large area of Central and West Asia (CAWA) and China require, in comparison to the Ukraine, Russia or northern United States, bread wheat cultivars with modest cold hardiness. Many widely grown bread wheat cultivars in CAWA are of a facultative type, often derived from crosses involving spring and winter wheats. These cultivars generally grow faster in spring compared to very cold-hardy cultivars, an advantage when terminal drought and heat may affect grain yield. However, during years with mild winter conditions, selection for facultative response under field conditions may not be effective, with the selection of spring-habit types favoured. Since prostrate growth is linked to the genes *Vrn1* and *Fr1*, genes which control vernalisation requirement and frost tolerance, respectively, the Turkey/CIMMYT/ICARDA International Winter Wheat Improvement Programme (IWWIP) screens all advanced lines for growth habit at two diverse locations. Winter survival data from international and national nurseries were analysed to evaluate a) the cold hardiness of spring × winter derived cultivars and b) the cold hardiness of cultivars grouped by growth habit. The cold hardiness of the best spring × winter derived cultivars was equal to that of cultivars derived from winter × winter crosses. Growth habit clearly separated winter-hardy from winter-tender types. The correlation between growth habit and winterkill (r=0.77**) suggests that growth habit can be used as an alternative trait for selection for cold tolerance in years with mild winters, particularly for those wheat breeding programmes which utilise spring × winter wheat crosses.

Key words: wheat, winter hardiness, winter × spring crosses, growth habit, frost tolerance

Introduction

Of the 220 million ha sown to wheat worldwide, 75 million ha are devoted to winter and facultative habit types of which about 25 million ha are grown in developing countries. Compared to areas with very severe winters such as the northern Great Plains of North America, the Ukraine or Russia, winter conditions in Central and West Asia (CAWA) and most parts of China are generally milder, though equally long in duration. Winter survival depends principally on the cold tolerance of the cultivars, but other factors are also important, in particular autumn stand establishment and rapid spring recovery and growth. Since it is commonly observed that wheat cultivars with high cold hardiness require longer periods for vernalisation requirement, have a longer dormancy period and exhibit slower growth in early spring (Fedulov, 1997), selection for more rapid growth and development in spring to promote higher yields often favours the selection of less winter-hardy types. The development of very winter-hardy wheat cultivars with fast development in spring may be difficult, since winter-hardy wheat cultivars tend to have a smaller cell size (Limin and Fowler, 1994). However, Richards (1996) showed that embryo- and cell-size are positively correlated with root growth and early crop development. Barley, which has a bigger embryo than wheat, grows much faster in spring due to more rapidly expanding cells, which may also partly contribute to the lower winter hardiness of barley compared to wheat. The importance of rapid spring growth is one reason why many widely grown wheat cultivars in winter wheat areas in developing countries are facultative, though with adequate winter hardiness for the target areas and often derived from spring × winter wheat crosses.

The measurement of winter survival in the field is still the standard procedure for most winter wheat breeding programmes, although freezing stress and consequently winterkill is probably among the most researched environmental stresses, both physiologically and genetically (Blum, 1988). The reasons for this have been discussed by Fowler et al. (1993). For breeding programmes located in areas which frequently experience mild winters, such as the IWWIP in Turkey, differential selection for winter survival in such years is not possible. For populations derived from spring × winter crosses there is even the risk of a shift towards more winter-tender types, which are often of a spring habit type.

Winter survival is a very complex trait and various authors (see Sutka and Snape, 1989) reported that at least 10 of the 21 pairs of wheat chromosomes are involved in the control of frost tolerance and winter hardiness, with chromosomes 5A and 5D being principally responsible. However, only one gene for frost tolerance, *Fr1*, has been identified to date, initially thought to be completely linked to *Vrn1* (Sutka and Snape, 1989). Galiba et al., (1995) found that the *Vrn1* and *Fr1* loci were closely linked on chromosome 5AL, but observed recombination. Since Roberts (1990) reported that *Fr1* and *Vrn1* are closely linked with prostrate growth type, the selection of cold-tolerant genotypes based on their growth habit should make it possible to overcome some of the problems related with insufficient differentiation in mild winters. As pointed out, this is particularly applicable for wheat breeding programmes utilising spring × winter crosses.

The winter survival rate of a wheat cultivar is not only dependent on its ability to survive low temperatures, but is also affected by various environmental and agronomic factors (Fowler and Gusta, 1978). Recently, zinc deficiency was identified as a major constraint for winter wheat production on the Central Anatolian Plateau (CAP) in Turkey (Cakmak et al., 1996). Zinc plays a crucial role in controlling gene expression, has important functions in cell wall structures and is an anti-oxidant. Data (Yildirim, 1996, unpublished) from 3 locations on the CAP indicate that autumn application of Zn fertiliser to

Bezostaya increased the number of plants/m^2 by more than 10%. For the IWWIP the main breeding task is not necessarily to enhance the cold tolerance beyond that of presently grown cultivars in the target area but to maintain a similar level of winter hardiness in spring × winter wheat crosses.

The objective of this paper is a) to present the results from a questionnaire on the importance of cold hardiness for wheat breeding programmes worldwide and b) to investigate how far growth habit can be used to select winter and facultative habit wheats with sufficient cold hardiness for winter wheat growing areas in Central and West Asia.

The importance of winterkill worldwide

In 1996, a questionnaire was distributed to 115 winter and facultative wheat breeding programmes worldwide, which had previously received the Facultative and Winter Wheat Observation Nursery (FAWWON), to collect information on breeding priorities, concern on genetic variability for further germplasm enhancement, breeding constraints and acreage, and wheat growing conditions in their target areas. Data were returned by 76 cooperators, addressing a total of 66.7 million ha. In this paper only data referring to winter survival are presented.

Table 1 gives the geographical distribution of the survey respondents and the ten-year frequency in which winterkill caused losses in farmers' fields. Based on this survey, winterkill damages wheat crops in farmers' fields in more than one out of ten years mainly in the United States, the Ukraine and Russia, whereas in large areas of West Asia and North Africa, Central Asia and most of

Table 1

Responses received by region and frequency of years within a ten-year period in which winterkill causes losses in farmers' fields

Region	No. of		Frequency out of 10 years		
	Responses	Countries	2 or less	3–5	6–10
Central Asia	9	6	5	3	1
West Asia and North Africa	11	6	8	2	1
Russia and Ukraine	4	2	1	2	1
East Asia	5	2	2	1	2
East and Central Europe	12	9	9	3	–
West Europe	17	8	14	2	1
North America	14	2	5	8	1
South America	3	3	3	–	–
South Africa	1	1	1	–	–
Total	76	39	48	21	7

Europe winterkill does not cause frequent damage. Considering that the estimated losses in years when winterkill occurs seldom exceed 20% (Table 2), and that these losses are mostly in areas where winterkill occurs frequently, these survey data suggest that the wheat varieties developed by the responding programmes mostly have sufficient winter hardiness for the areas targeted. Only three out of the 76 respondents reported winter hardiness as the most important breeding trait in their programme. Similarly, Fowler et al. (1993) concluded that plant breeders have successfully maintained the cold hardiness levels of cultivars for their target areas. Furthermore, estimates of economic losses may be higher than the actual plant death, due to the ability of wheat to compensate by tillering, albeit within limits.

Table 2

Economic losses due to winterkill in farmers' fields in years when winterkill occurs and number of wheat breeding programmes observing severe winterkill damage for Bezostaya in some years

Region	No. of programs	Economic losses in years with winterkill (as %)				No. of programmes reporting severe winterkill in some years for Bezostaya
		<10	10–20	21–50	> 50	
Central Asia	9	2	3	3	1	1
West Asia and North Africa	11	8	2	1	0	0
Russia and Ukraine	4	0	2	2	0	1
East Asia	5	2	2	1	0	1
East and Central Europe	12	4	7	1	0	0
West Europe	17	10	4	3	0	1
North America	14	4	8	2	0	5
South America	3	2	1	0	0	0
South Africa	1	1	0	0	0	0
Total	76	33	29	13	1	9

The actual level of winter hardiness required for most winter wheat growing areas does not exceed that of Bezostaya 1, since 61 out of 71 respondents observe no or only slight winterkill in this variety (Table 2). However, in some major winter wheat growing areas in Russia, the Ukraine, the United States and Canada, the winter hardiness level of Bezostaya 1 is not sufficient, as was also shown by Fowler and Gusta (1978).

Results for winter survival of spring × winter crosses

Three sets of data were used to compare the winter hardiness of bread wheat cultivars derived from spring × winter crosses with that of winter × winter crosses and to investigate the suitability of growth habit as a selection criterion for cold tolerance in years with mild winters.

- Data Set 1 consists of the winter survival data of 210 entries in the 5th FAWWON from 19 locations. Detailed information on the locations in the fifth FAWWON and the winterkill of each entry at each location has been published elsewhere (Anonymous, 1997). To evaluate growth habit, the entries were sown in late spring in Moscow, Russia; Ankara, Turkey; and Aleppo, Syria. Scores were given on a 1 to 5 scale. Entries with prostrate growth and vernalisation requirement were scored as winter wheats. The entries in the 5th FAWWON originated from sixteen winter wheat breeding programmes in the US, Eastern Europe, the Ukraine, Iran, Turkey and Syria. The spring wheat check, Seri 82, was excluded from the analysis.
- Data Set 2 consists of field scores for the winter survival of 674 entries in the replicated yield trials of the IWWIP in 1996/97 from Eskisehir, Ankara and Konya, Turkey. Entries were scored in early spring on a 1 to 5 scale where 5 indicated complete kill of the respective entry. The scores for growth habit of entries in this data set were obtained from Izmir, Turkey.
- Data Set 3 consists of the survival rate of 240 bread wheat cultivars in the 1995/96 yield trial of the IWWIP exposed to –14°C. The screening was done by V. Vlassenko at the Mironovska Institute, Ukraine. Entries were vernalised for 45 days and then kept for 6 days at 0°C, for 1 day at –2°C and for 3 days at –4°C. The temperature was then lowered by 2°C per hour and the entries were exposed to –14°C for 24 hours. Surviving plants were counted three weeks later.

Winter survival of the 5th FAWWON entries

The mean winter survival rating across 19 locations for entries with winter, facultative and spring habit was 77%, 63% and 38%, respectively. Sixty-four entries tested in the 5th FAWWON were derived from winter × spring wheat crosses. Growth habit tended to separate entries with high winter survival from those with low survival (Fig. 1). Four entries did not follow this separation. Two entries with spring habit, KS82142/Seri and CO724377/Nac//Seri, had winter survival rates of 85% and 76%, respectively, which is comparable to that of winter wheats. Two other entries, both from Iran, were classified as winter wheats but exhibited low winter hardiness. These data, and the highly significant correlation between growth habit and mean winter survival (r=0.77**) suggest that breeding programmes utilising spring × winter crosses to develop wheat cultivars for areas which require medium winter hardiness may use growth habit as a selection criterion, particularly in years when mild winters do not differentiate between winter-tender and winter-hardy cultivars. Furthermore, in years with mild winters, there is a tendency to select for spring habit types, since in general, spring types are more vigorous. If

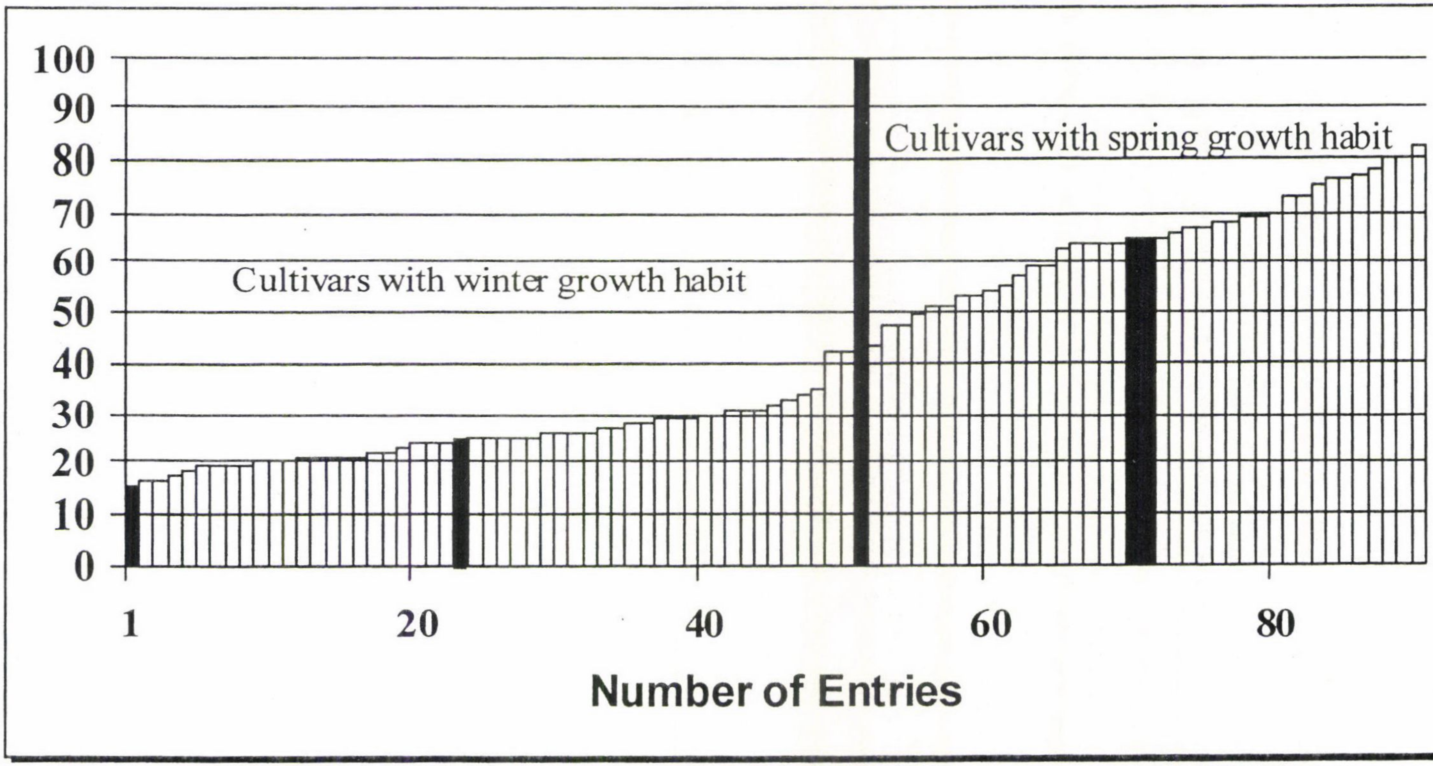

Fig. 1. Winterkill (%) of cultivars in 5th FAWWON derived from winter × spring crosses. Left of the black bar are cultivars with winter growth habit, to the right cultivars with spring growth habit. The exceptions within each group are marked with black bars.

terminal drought or heat stress during grainfill limits grain yield, spring types tend to give higher grain yields than true winter wheats. In the case of a repeated series of mild winters, there is a risk of selecting against winter-hardy types and of shifting breeding populations towards cold-tender spring types.

For the entries tested in the 5th FAWWON, representing wide genetic diversity, locations with an average winterkill rating between 25% and 40% proved to be most suitable. For these locations, the average correlation between winterkill rate at each location and mean winterkill across locations varied between 0.80** and 0.92** (Table 3). These correlations are high considering the problems associated with accurate field scoring and screening. That winterkill affected grain yield is shown by the highly negative correlation between winterkill and mean grain yield (Table 3). Locations with higher or lower winterkill rates were not as suitable since they did not differentiate the germplasm well.

Table 3

Correlation between winterkill at each location (WK-LOC) and winterkill across 19 locations (WK-MEAN) of 210 bread wheat cultivars included in the 5th FAWWON and between WK-LOC and grain yield (GY-Mean) across locations. Mean WKILL is the average winter kill rate of all entries at each location

Location	Correlation between WK-LOC and		Mean winterkill as %
	WK-Mean	GY-Mean	
USA, Arkansas	0.92	–0.79	36
Ukraine, Kharkov	0.92	–0.79	28
Lithuania, Kedainia	0.91	–0.82	40
Japan, Kitami	0.90	–0.81	29
Japan, Iwate	0.89	–0.78	27
Poland, Leszno	0.88	–0.72	35
USA, New York	0.87	–0.72	38
Denmark, Zealand	0.87	–0.75	30
Canada, Charlottetown	0.80	–0.68	23
Czech Rep., Stupice	0.76	–0.67	39
Ukraine, Crimea	0.74	–0.69	29
Turkmenistan, Tahal	0.69	–0.61	21
Poland, Radom	0.62	–0.45	41
Ukraine, Kievskaya	0.49	–0.40	28
Ukraine, Odessa	0.47	–0.45	2
USA, Minnessota	0.42	–0.22	90
Ukraine, Kiev	0.22	–0.16	54
Croatia, Zagreb	0.12	–0.23	15
Afghanistan, Wardak	0.10	0.19	16

Table 4
Mean, minimum and maximum winterkill (%) of cultivars in 5th FAWWON originating from winter wheat breeding programmes in Europe, USA and Asia. Data are given as average across 19 locations which reported winterkill

Cultivar, per se or cultivar programme of origin	No. of occurances or cultivars	Winterkill (%)		
		Mean	Min.	Max.
Bezostaya (Cultivar)	3	13	8	19
Local Check	4	14	9	20
Ukraine	7	20	18	26
USA other than Great Plains	11	20	14	25
Hungary	6	21	17	28
Nebraska, USA	11	22	16	26
Kansas, USA	20	22	14	35
Gerek (Cultivar)	3	22	19	25
Turkey	18	23	15	50
Bulgaria, Tolbuhin	17	26	18	46
Oregon/Mexico/CIT	27	35	15	76
Bulgaria, Sadovo	8	35	16	70
Romania	8	35	17	70
Syria	12	45	21	82
Atay (cultivar)	3	49	47	51
Iran	2	61	59	63
Seri (Spring wheat check)	3	74	67	82

The performance of germplasm from different breeding programmes is shown in Table 4. Germplasm from the Ukraine, Hungary and the Great Plains in the USA had the lowest average winterkill ratings as a group, varying from a minimum of 20% for an entry from the Ukraine to a maximum of 35% for an entry from Kansas. Entries originating from programmes in Bulgaria, Rumania and the IWWIP, which includes lines from Mexico, Oregon and Turkey, had an average a higher winterkill rate across locations. The winterkill rate of Romanian lines was relatively high, since mainly facultative wheat cultivars were included. Rumanian winter wheats tested in previous FAWWONs had a winter survival rate similar to that of Hungarian cultivars. However, the best entries from each group had similarly low winterkill ratings. The lower frequency of winter-hardy entries from these programmes is a reflection of the less severe winters in their selection target environments.

The most winter-hardy entry was the check cultivar Bezostaya, with an average winterkill rate of 8 %, followed by the "local check" with 9%. At each location, other entries showed winter survival ratings equal or better than Bezostaya. This can be expected, since Veisz (1991), summarising data from genetic studies in the phytotron, concluded that frost resistance is always relative. There were no entries which were resistant under all conditions or environments, since the development, maintenance and breakdown of frost resistance is controlled by both genetic and environmental factors. The wide adaptation of Bezostaya may be partly due to its ability to cope with the wide range of stresses occurring in cold environments, whereas other genotypes are more specifically adapted to cold stresses occurring in either humid or dry areas. Similar results were obtained in previous FAWWONs. In five out of the last seven FAWWONs the winter hardiness level of Bezostaya was as good as that of the most winter-hardy entry from winter wheat breeding programmes in the USA, Asia and Europe (Table 5).

Table 5

Winter kill (%) for best (least) and worst entry and for Bezostaya, local check, and the best winter × spring derived cultivar in the 1st–5th FAWWON and the 5th–6th IWWSN

Nursery and year distributed	Best cultivar	Worst cultivar	Bezostaya	Local check	Best W × S cultivar	No. of sites
5th FAWWON 95/96	8	82	8	9	11	19
4th FAWWON 94/95	1	47	4	5	3	5
3rd FAWWON 93/94	24	96	25	32	32	5
2nd FAWWON 92/93	3	55	4	19	8	10
1st FAWWON 91/92	9	43	9	17	12	8
6th IWWSN 90/91	18	50	25	19	21	8
5th IWWSN 89/90	5	86	5	–	5	6

Winter survival of genotypes grouped by growth habit

The average, minimum and maximum scores for frost tolerance of 674 bread wheat genotypes from Ankara, Cumra and Eskisehir, Turkey are given in Table 6. By contrast to the 5th FAWWON, all entries originated from selections carried out in Turkey within the IWWIP. None of the 302 entries with a winter or facultative/winter growth habit had a score worse than 3, which was similar to the average score of 2.8 for facultative/spring and 3.2 for spring types (Table 6). The correlation between growth habit in a spring wheat environment (Izmir) and frost tolerance scores was highly significant (r=0.67**). From this data it can be concluded that growth habit is a suitable trait for use in the IWWIP to select for frost-tolerant cultivars.

Table 6

Mean, minimum, maximum and standard deviation (SD) of scores for frost tolerance of 674 advanced bread wheat cultivars. Data are from Ankara, Eskisehir and Cumra, Turkey in 96/97. Scores are averaged across locations

Growth habit*	Entry No.	Score for frost tolerance**			
		Mean	Min.	Max.	SD
Winter	210	1.6	1.0	3.0	0.50
Facultative/Winter	92	2.1	1.0	3.0	0.52
Facultative	188	2.2	1.0	3.7	0.69
Facultative Spring	99	2.8	1.3	5.0	0.60
Spring	85	3.2	1.3	5.0	0.70
Total	674	2.2			

*Growth habit was scored as 1 to 5 with 1 = winter habit and 5 = spring habit

** Frost tolerance was scored on a 1 to 5 scale with 5 = killed

Winter survival of advanced lines in the IWWIP at –14°C

The survival rate at –14°C of 240 advanced lines from the IWWIP and of Turkish cultivars, which were included as checks, is given in Table 7. The mean survival rate of cultivars grown in Turkey varied between 51% for Gun 91, and 66% for Bolal and Bezostaya. However, the difference between the lowest and highest survival rate of each check, varying from 22% for Dagdas to 55% for Gerek, indicates the need for parallel field screening. Fowler (1981) reported that results from controlled environments are often less repeatable than results obtained in field screening for cold hardiness. The survival rate of spring × winter derived genotypes was on average smaller than that of winter × winter derived crosses, though the best entries had a survival rate equal to that of Bezostaya. As expected, the frequency of cold-tolerant genotypes derived from winter × winter crosses is much higher, since they all carry *Fr1*.

Table 7
Mean, minimum and maximum survival rate (as %) at –14°C for 24 hours of wheat cultivars grown in Turkey and lines derived from winter × spring, (winter × spring) × winter and winter × winter crosses

Cultivar/ Type of cross	No. of readings	Survival rate (%) after 24 hours at –14°C		
		Mean	Min.	Max
Bezostaya 1	7	66	36	82
Bolal	7	66	34	87
Gerek 79	10	54	32	87
Seri 82 (Spring wheat check)	4	10	0	20
Gün 91	8	51	31	77
Dagdas	4	59	49	71
Winter × spring	66	37	0	83
(Winter × spring) × winter	108	40	0	90
Winter × winter	66	58	5	100

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DEVELOPMENT OF YOUNG MAIZE PLANTS UNDER A SUBOPTIMAL RANGE OF TEMPERATURES

C. L. MARTON and T. SZUNDY

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, P.O. BOX 19, HUNGARY

Six genetically diverse inbred lines with different levels of cold tolerance were investigated at 10 different temperatures from 9°C to 18°C at 1°C intervals for 33 days. There was a significant difference between the inbred lines as regards the cold tolerance of young plants. The cold tolerance of the inbred lines gave different rankings according to the plant trait evaluated. A 654 and HMv 16 produced the most dry matter and F2 and HMv 307 the least. A very similar ranking was found for final plant height, leaf area and fresh shoot weight. The dry matter content of the young plants was about 10% at temperatures above 13°C. At lower temperatures (9–12°C) the dry matter content of ČM 174 was the highest, followed by HMv 307 and HMv 16. An evaluation of the weekly increment in plant height at 9–12°C showed that F 2 had the longest period of growth, while Mo 17 stopped growing earliest.

Key words: maize, cold.tolerance, cold test, gradient chamber

Introduction

The cold tests elaborated by breeders and geneticists consist basically of a combination of two temperatures: cold incubation and germination. These methods are suitable chiefly for the evaluation of cold tolerance during the germination stage. The evaluation of seed vigour provided by the cold test is greatly influenced by other external factors such as seed dressing, the duration of the cold treatment, the temperature applied and the germination medium. Due to these deficiencies numerous other methods for testing seed vigour have been elaborated, including accelerated aging (Delouche and Baskin, 1973), the tetrazolium test (Moore, 1962), the examination of the rate of germ growth (Burris et al., 1969) or of germination (Kotowski, 1926), the mechanical injury test (Koehler, 1957; Simak and Kamra, 1963) and the electrical conductivity test (Mathews and Bradnock, 1968). Although these methods partially eliminate the deficiencies of the cold test, they are restricted to the evaluation of cold tolerance at emergence and give no information on the effect of cold stress in the young plant stage.

While methods serving to evaluate cold tolerance in the germination stage are mainly limited to an evaluation of percentage emergence and emergence date (Szundy, 1981), Mock and Skrda (1978) also recorded the dry weight of 42-day-old plants and Hoard and Crosbie (1985) the dry mass of the germ. In their studies, however, both the young plants and the germinating seeds were

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given cold treatment, which meant that the cold tolerance at emergence and in the young plant stage could not be separated.

A new approach was proposed by Gupta and Kovács (1976), who exposed seedlings to a short "cold-wave" treatment after emergence.

In the present experiments a similar method was employed, making use of the advantages of the gradient chamber, where inbred maize lines could be exposed to ten different temperatures simultaneously.

Materials and methods

The examinations were carried out on 6 genetically diverse inbred lines with different levels of cold tolerance (Table 1). The seeds of these lines were previously germinated at 18°C in a GB phytotron unit and the pots were transferred to the gradient chamber when the plants reached the 2-leaf stage. In the gradient chamber (Tischner, 1989) the day temperature was programmed in 10 rows between 9 and 18°C, at 1°C intervals, while the night temperature in each row was 9°C. The intensity of illumination was 30 klx. The experiment was carried out in two replications. The results were evaluated 33 days after transfer to the gradient chamber. Leaf area, leaf number, green shoot mass, dry shoot mass and shoot dry matter percentage were determined at the end of the experiment, while plant height was measured weekly from the beginning of the experiment. The data were evaluated using two-factor variance analysis (Sváb, 1981). When compiling the figures moving averages (average of 3 temperatures) were used to characterise the genotypes. For ease of interpretation, only lines with characteristic behaviour are included in the figures.

Table 1
List of the inbred lines tested

Line	Origin	Cold tolerance*	Kernel type
A654	A 116 × WF 9	sensitive	dent
Mo 17	187-2 × C 103	sensitive	dent
Hmv 16	FR 19 × CM 105	tolerant	dent
Hmv 307	P 3709 SC	medium	dent
F 2	OP. Lacaune	tolerant	flint
CM 174	V 3 × B 14^3	tolerant	dent

* According to previous observation in Martonvásár

Results and discussion

In the course of the experiment considerable growth was observed at all temperatures, but at the highest temperature the plants were 3–4 times taller than at the lowest temperature (Fig. 1).

At all temperatures CM 174 was the tallest and F 2 the shortest. The growth of A 654 was similar to that of CM 174. CM 174 was also ranked first for the increase in the number of leaves (Fig. 2). At low temperature (9–13°C) F 2 gave equally good values, but at higher temperatures F 2 was one of the slowest developers, similar to Mo 17, which, however, also developed very

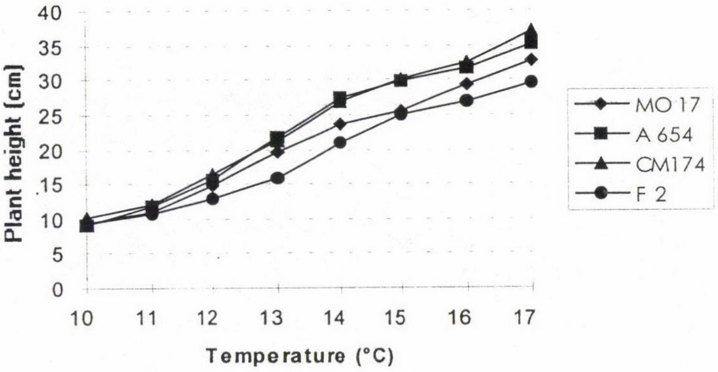

Fig. 1. Plant height of the inbred lines

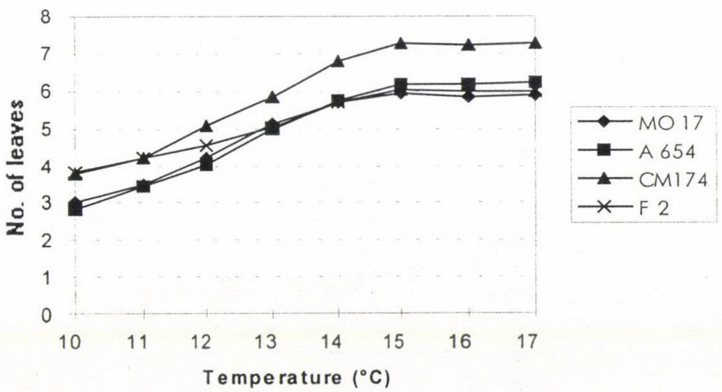

Fig. 2. Number of leaves of the inbred lines

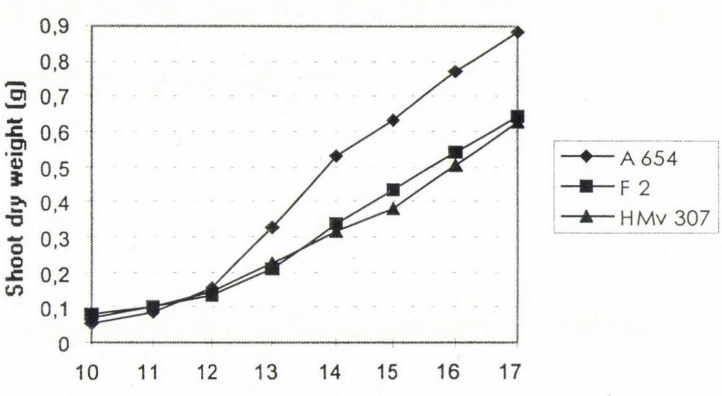

Fig. 3. Shoot dry weight of the inbred lines

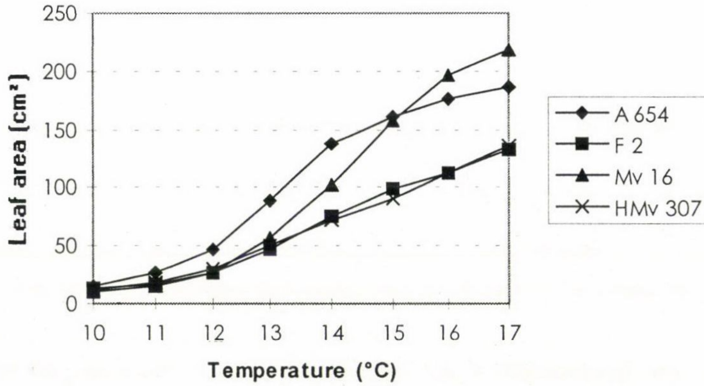

Fig. 4. Leaf area of the inbred lines

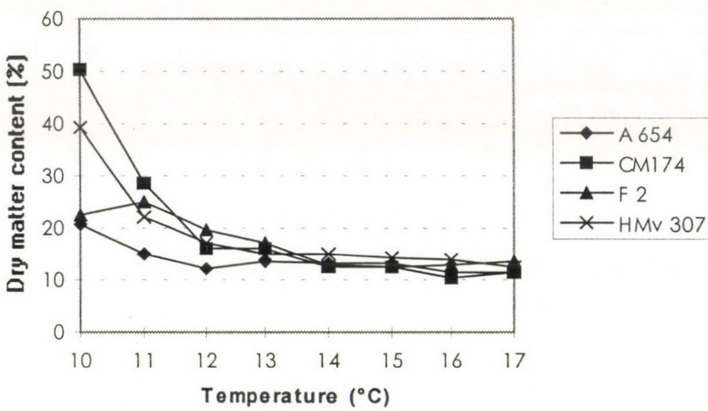

Fig. 5. Dry matter content (%) of the inbred lines

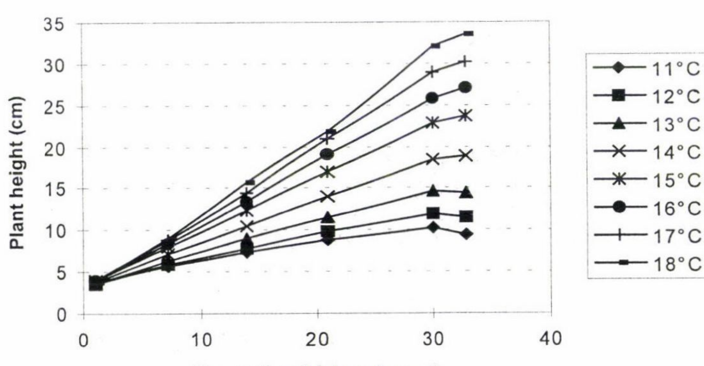

Fig. 6. Changes in plant height during the cold treatment

slowly in the cold. The dry mass of the shoots was similar for all the lines below 13°C (Fig. 3), while at higher temperatures A 654 produced the greatest shoot dry mass and F 2 the smallest. A 654 produced the largest and F 2 the smallest leaf area (Fig. 4) at all temperatures, while HMv 307 was comparable to F 2. A similar cold tolerance ranking was obtained for the lines with all the above characters: A 654 was found to be good on the basis of all the characters, and HMv 16 and ÇM 174 for most characters. Mo 17 was average, while F 2 and HMv 307 generally gave poor results.

The shoot dry matter percentage gives an indication not of the growth over a given period, but of the survival ability at different temperatures. Under normal conditions the dry matter percentage in young plants is around 10%. Values similar to this were exhibited by the tested lines at temperatures above 13°C (Fig. 5). Below this temperature, however, there was a considerable drop in moisture content, to a varying extent for each line. The desiccation of CM 174 was the most intense, followed by HMv 307 and HMv 16. In terms of the moisture content of the shoot, A 654, Mo 17 and F 2 retained their viability to the greatest extent and for the longest period in the course of the 33-day cold treatment. The ranking obtained on the basis of shoot dry mass percentage was thus quite different to that observed for the other characters.

The responses given to various temperatures were also evaluated by studying changes in plant height as a function of time. The data are presented in Fig. 6 over the average of the lines. At above 12°C the plant height increased continually in time, while below 12°C the rate of increase declined, and finally the plant height itself was reduced. For each inbred line a quadratic function was fitted to the average values of plant height as a function of time at temperatures of 9, 10 and 11°C. The "x" values belonging to the maximum values of the parabola were calculated. These indicate on which day of the cold treatment each line reached maximum plant height, after which its height was reduced.

Continued growth was indicated for the longest period for F 2, which was predicted to reach maximum plant height on the 39th day (Table 2).

Table 2
Maximum values of plant height of the inbred lines during cold treatment
(average of 9–11°C)

Inbred lines	Function	Average of 9–10–11°C	
		x max. (day)	y max. (cm)
Mo 17	$y = 4.2+0.4x-6E6.7x^2$	29	10
A 654	$y = 2.7+0.4x-6E7.0x^2$	31	9
CM 174	$y = 4.4+0.4x-6E4.8x^2$	36	11
F 2	$y = 2.9+0.4x-6E4.6x^2$	39	10
Hmv 16	$y = 2.9+0.4x-6E6.1x^2$	34	10
Hmv 307	$y = 3.3+0.4x-6E6.4x^2$	32	10

This was followed by CM 174, which would achieve maximum plant height on the 36th day. Since the treatment was only carried out for 33 days, these values must be regarded with certain reservations, as is true in general in biology when values based on mathematical functions are extrapolated beyond the range studied.

These reservations are confimed by the trend in shoot dry matter percentage for CM 174, which averaged 48.21% in the 9–11°C range, with an extreme valuė of 87%. This means that the majority of the CM 174 plants were destroyed and were incapable of further growth even on the 33rd day. The dry matter percentage of F 2 in the same temperature range was 26.14% and even the highest value was only 33.5%.

Studies on the duration of growth, or the ability to grow, thus lead to a different order of lines than that found for the characters previously considered, showing that this is another form of cold tolerance. The data presented above demonstrate that the cold tolerance of the lines changes in the course of development: the genetic background of cold tolerance is different at emergence than in the young plant stage. Two forms of the latter can be distinguished. The first, embodied by A 654 and HMv 16, is characterised by rapid growth lasting for 3–4 weeks. The second, represented by F 2, survives long periods of cold not through the intensity of growth, but by retaining the ability to grow.

These two types of cold tolerance are adapted to different climatic conditions. In a continental climate, such as that experienced in Hungary, there is usually a rapid rise in temperature after sowing. If a cold spell occurs it rarely lasts longer than 1–2 weeks. In such a climate the best genotype is one which is capable of fairly intensive growth even during the cold wave (Gupta and Kovács, 1976).

In an oceanic climate, however, the spring temperature rise is slower and the cold spell may last longer. Here, in addition to growth intensity, the ability to continue growth and retain viability becomes an important part of cold tolerance.

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BREEDING FROST-RESISTANT WHEAT VARIETIES USING THE PHYTOTRON FACILITIES

L. LÁNG, Z. BEDŐ and O. VEISZ

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, H-2462, MARTONVÁSÁR, P.O. BOX 19, HUNGARY

Wheats grown in Hungary must be capable of surviving cold continental winters without snow cover. The frost resistance of the varieties must be at least as good as that of the old cultivated variety Bánkúti 1201, and if possible should approach or equal that of Bezostaya 1. Field selection is not always efficient, so phytotronic frost tests are of great significance. Due to the restricted testing capacity, wheat breeding in Martonvásár is based on the screening of crossing partners and the testing of advanced lines. By producing dihaploids it is possible to select efficiently for frost-resistant types in early generations. As a result of this selection procedure the Martonvásár wheat varieties possess good frost resistance, which means that they can be grown even in countries with winters colder than those experienced in Hungary.

Key words: wheat, breeding, frost tolerance, winter hardiness

Introduction

In Hungary the minimum daily temperature from early December to late February, averaged over a long period, tends to be constantly below freezing point. The absolute minimum temperature measured on any day during this period is always below –10°C. The coldest weather is usually recorded in mid-January, when temperatures of –35°C or below have often been measured. The milder weather sometimes occurring in early February is frequently followed by a further cold spell. Low winter temperatures without snow cover cause damage of economic proportions in winter wheat every 15–20 years, but the potential danger must be faced every year. In Central Europe the most important factor of winter hardiness is frost resistance. Breeding for winter hardiness is complicated by the fact that the winters are frequently mild and the cold is often accompanied by snow cover, in which case genotypes with poor frost resistance, or even spring wheats, are able to overwinter. For this reason, one of the first research fields studied in the Martonvásár phytotron after it was opened in 1972 was the frost resistance of wheat and the elaboration of an objective testing method.

Materials and methods

In the frost resistance testing method described by Veisz and Tischner (1997) the plants are raised in wooden boxes with internal dimensions of 38 cm × 26 cm × 11 cm. The growth medium is a 4:1 mixture of garden soil (chernozem with forest residues) and sand. The plants are grown in nine rows, with 20 plants per row. Each box represents one replication, with four replications per experiment. The M29 climatic programme (Tischner et al., 1997) applied during the six-week preliminary growth period involves a weekly lowering in the temperature accompanied by a shortening of the daylength, similarly to the autumn weather conditions in Hungary.

The preliminary growth takes place in PGV-36 chambers, as does the first phase of hardening, when the temperature fluctuates daily between 3°C and –3°C for a week. The second phase of hardening, which lasts for four days at –4°C, and freezing at –15°C for 24 hours are carried out in a Conviron C-812 frost testing chamber. For further growth and evaluation the plants are transferred to a GB-48 phytotron unit.

Results and discussion

Under Hungarian climatic conditions the critical level of frost resistance can be determined accurately in terms of known varieties. The wheat variety Bánkúti 1201 was grown for around 50 years. In the majority of cases it overwintered without damage, though during colder than average winters some or all of the plants were sometimes destroyed. The frost resistance of this variety is now regarded as a minimum criterion, corresponding to moderate frost resistance compared to the best varieties now available. The most widely-grown variety during the next period was Bezostaya 1, which had satisfactory frost resistance and winter hardiness even in snowless winters. The results presented in Table 1 illustrate how the frost resistance values of certain well-known varieties compare to that required in Hungary.

In general wheat does not need to be more frost-resistant than Bezostaya 1, so the breeding aim is to develop varieties whose frost resistance is of this standard, but certainly no poorer than that of Bánkúti 1201.

Table 1
Frost resistance of varieties of different origin compared to the level required in Hungary

Variety	Origin	Survival in frost test (%)
Siouxland	USA	96
Martonvásári 9	Hungary	95
Obriy	Ukraine	95
Bezostaya 1	Russia	87
Partizanka	Yugoslavia	79
Monopol	Germany	69
Kavkaz	Russia	64
Bánkúti 1201	Hungary	53
Thesee	France	36
Fundulea 4	Romania	31
Maris Huntsman	UK	22
Etoile de Choisy	France	5
Soissons	France	1
Libellula	Italy	0

The ability to select wheat varieties with satisfactory frost resistance was created by the phytotronic frost test elaborated in Martonvásár (Sutka, 1981; Veisz and Tischner, 1997; Tischner et al., 1997). Each experiment involves 64 varieties, which means that 200–400 genotypes can be tested each year. In each case three control varieties are included, one of which is frost-resistant (Mv 4), one moderately resistant (Bánkúti 1201) and one frost-sensitive (NS Rana 2). The losses recorded in each experiment for the standards range from 0–5 to 80–95%, while the experimental error ($LSD_{5\%}$) is 10–20%. In repeated frost tests the frost resistance values measured for the same variety may differ by ±10–15%, but the order of the varieties and the way their frost resistance compares to the controls can be reliably reproduced.

The breeding method must adapt to the restricted testing capacity, so examinations are chiefly made to evaluate crossing partners and to check the frost resistance of advanced lines.

In most cases the varieties used in crosses are previously tested for frost resistance. Varieties of various origins tested over the last 15 years exhibit a wide range of genetic variability both globally and within sites of origin (Table 2).

Although there is a characteristic mean frost resistance level for wheat varieties originating from a given country, in most regions varieties with both poor and good frost resistance are bred. On the basis of freezing data, frost-resistant × frost-resistant single cross combinations are generally created. If a genotype with poor frost resistance is required as a parent for any reason, a satisfactory level of frost resistance is ensured by means of top-crossing or double crossing.

During the first 6–8 years following crossing, selection is carried out under field conditions. Throughout the breeding period the majority of varieties are never exposed to really cold, snowless winters which would assist selection.

Table 2
Frost resistance of varieties of different origins

Country of origin	Survival %		
	Average	Minimum	Maximum
USA	81	37	96
Romania	77	57	96
Ukraine	75	14	98
Canada	70	54	86
Slovakia	59	6	99
Germany	54	32	73
Bulgaria	49	0	96
Croatia	33	9	63
Turkey	26	6	80
Yugoslavia	26	0	79
France	23	1	59
UK	15	0	55

There are generally 1–2 winters which cause a small extent of frost damage, sufficient to identify the genotypes with the poorest winter hardiness. It is thus extremely important to check the frost resistance of every advanced line in addition to field selection. The results achieved over nearly 20 years indicate that around 90% of the advanced lines possess satisfactory frost resistance (Fig. 1). The frost tests carried out prior to state variety trials ensure that the entries from Martonvásár are always among the best in this respect.

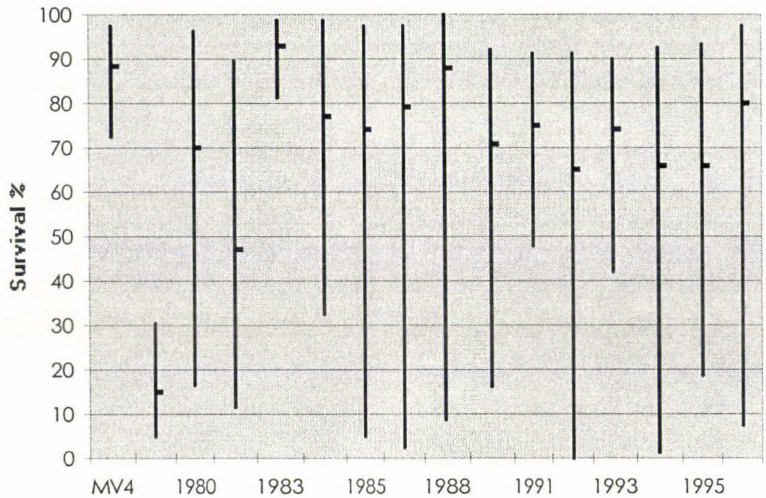

Fig. 1. Frost resistance of advanced Martonvásári wheat lines (minimum, maximum and average values)

One special application of the frost test is the reselection of lines with excellent traits but only moderate frost resistance in the course of variety maintenance. During this variety maintenance significant differences are often observed between the sublines, which means that selection based on the phytotronic frost test leads to the development of a variety with reliable frost resistance. The phytotronic frost test also provides valuable data for the selection of parents for hybrid wheat and for the evaluation of the F_1s.

When selecting lines originating from crosses between winter and spring genotypes, frost resistance testing is especially important. In this respect the majority of lines do not satisfy even the minimum requirements for Hungary, but this only becomes obvious during the frost test after several years of selection and trials. The long years of expensive field experiments can be considerably reduced by combining the advantages of anther culture-derived dihaploid production and the phytotron frost-testing facilities. Sufficient seed (a

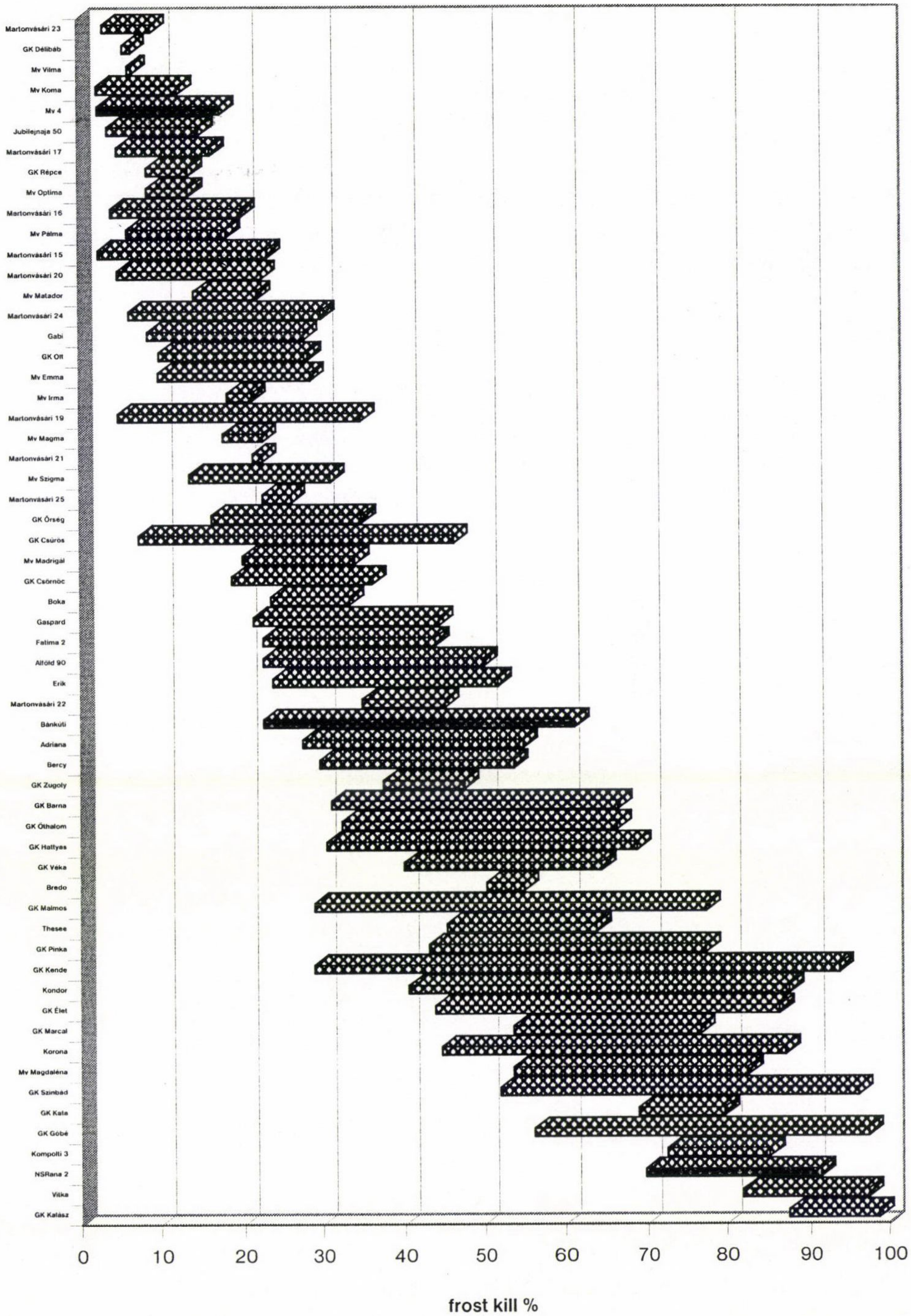

Fig.2. Frost resistance of registered wheat varieties in Hungary

minimum 200) are available for the frost test the year after dihaploid production, which means that field testing is limited to satisfactory genotypes. Using this method it is possible to eliminate the disadvantage of the frost test, which is not suitable for the selection of individuals, but gives a reliable evaluation of the frost resistance of uniform lines.

As the result of targeted selection for frost resistance, 18 of the 25 most frost-resistant winter wheat varieties registered in Hungary were bred in Martonvásár and most of these can be reliably grown in countries with an even colder climate (Fig. 2).

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FUTURE PROSPECTS OF PHYTOTRONICS

T. W. TIBBITTS

HORTICULTURE DEPARTMENT, UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN, 53706 USA

Phytotronics has been recognized for nearly half a century. It began with a few centralized facilities in Australia, the Netherlands and the United States and expanded quickly in the succeeding two decades with facilities in nearly every developed country of Europe and Asia including Hungary.

In the last decade, a large amount of phytotronic effort in the United States, Japan and Europe has developed an understanding of the role plants can play in life support in long-term space bases. This effort involves the use of closed chambers, and large closed facilities are being operated at the Kennedy Space Center in Florida, USA, at the Johnson Space Center in Texas, USA and at the Institute for Environmental Sciences near Aomori in Japan. This space-directed research has led to the development of some unique technologies including the use of small LED lamps, microwave lamps, watering and humidity control procedures using solutions under negative pressure, and photocatalytic removal of ethylene.

Attention will be given to emphasizing some of the significant problems that have reduced the effectiveness of phytotronic research over the years, such as excessive longwave radiation, chamber leakage, and volatile contaminants. The future needs for phytotrons in studying environmental degradation, indoor contaminant control, and for specialized crop production will be discussed

Key words: phytotronics, space technologies, closed environments, LEDs, microwave lamps

Introduction

Phytotronics has been a recognized science for only the past half-century. It had its beginnings in the early third of this century with the attempts to control plant growth in chambers constructed in greenhouses, supplementing the variable sunlight with lighting from tungsten lamps. However, these greenhouse chambers could not adequately control the lighting for the plants. Then came the phytotron activity in the late 1940s as the useful and cool fluorescent lamps became available. Initially, scientists contracted with air-handling engineers to convert or build rooms adjacent to their research laboratories for growing plants. Many ‘phytotronics’ experts emerged but there were more failures than successes in these converted rooms as scientists quickly realized that they were not duplicating the natural plant environment effectively and they were plagued with unexpected problems and contaminants in these new enclosed growth facilities.

From these experiences emerged a few real phytotronics experts, namely Chouard, Evans, Gaastra and Went, who had the expertise in both plant science and environmental engineering to design and construct facilities that grew plants effectively, and as a consequence they attracted scientists from the world over to

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their facilities to unfold the complex environmental interactions in plants. Large facilities were constructed in Australia, Canada, France, Germany, Japan, New Zealand, the Netherlands and the United States in the 1950's and 1960's, each with very unique and different designs providing effective controlled environments in research areas. This initiated two decades of excitement and scientific pre-eminence for environmental physiologists.

During this period, engineers in commercial companies saw the opportunities in this new research area and these commercial companies began designing and building self-contained plant growth units, requiring only electrical and plumbing attachments for operation. They were able to draw on information from the previous decade of testing and evaluation in research laboratories and phytotrons to construct chambers and rooms with a maximum of reliability and environmental flexibility. This then spawned a new generation of phytotron facilities developed around these commercial chambers. Thus in the 1960s and 1970s, many more large facilities were developed in Canada, Germany, Great Britain, Hungary, Japan, Poland, the United States and many other countries, built around commercial chambers and rooms of many different sizes and with varied capabilities for environmental control.

However, in many countries the availability of commercial growing units during this period also led to the diminishing utility of phytotrons, as centralized facilities in many countries for effective and reliable growth units could be purchased and located directly adjacent to research areas. This made it possible for small groups of scientists to build mini-pyhtotrons within their departments and institutes. Thus this use of chambers proliferated in countries where electricity and cooling capabilities were readily available. This caused a reduced demand for the environmental control facilities at centralized phytotrons.

Space facilities

The most recent activity in phytotronics is evolving around the need to grow plants for life support in space and requiring chambers that are tightly closed so that the solids, liquids and gases can be effectively recycled.

Plants have the unique capability of providing for the four major components of life support for humans in space; food, oxygen, water, and carbon dioxide removal. This life support has a large weight cost, for each day a person in space requires 0.6 kg dry food, 0.9 kg oxygen, 1.8 kg water to hydrate food, 2.3 kg water for drinking, 16.8 kg water for domestic use, and 1.1 kg of chemical to remove carbon dioxide. Plants have the capability of providing all of these requirements. They utilize carbon dioxide and release oxygen in the process of photosynthesis that produces useful foodstuffs, and the plants transpire water, utilizing waste water taken up by the roots. This transpired water can be condensed from the air as useful potable water.

The first large-scale closed facility for studying the use of plants for providing life support was constructed in Krasnoyarsk, Russia in the 1970s (Gitelson et al., 1976). A facility of 150 m^2 was constructed to provide life support for 3 persons enclosed for 6 months. Wheat was the principle crop maintained in this facility. This Russian experience led to efforts during the 1980s and 1990s in other countries, initially in the United States, but now extended to Japan, Canada and European countries. A large test chamber was retrofitted at the Kennedy Space Center in Florida in the 1980s with 20 m^2 of growing area for the study of plant growth in a closed facility (Prince et al., 1987). The chamber has provided a wealth of information on different crops when grown in a recycling mode. Of significant physiological interest is the response of canopies of plants over their complete life cycle from planting to harvest. They have documented the patterns of carbon dioxide exchange, transpirational water, oxygen evolution and ethylene release (Wheeler et al., 1993, 1996). The ethylene release curves are of particular interest for they have documented that ethylene production is closely proportional to the photosynthetic activity of the plants.

Additional closed facilities are being constructed to grow plants and simultaneously provide life support for humans. These are being constructed both at the Johnson Space Center in Houston, Texas, USA (Henninger et al., 1996; Tri and Henninger, 1995) and in Japan at the Institute for Environmental Sciences at Rokkasho-mura near Aomori in Japan (Ashida and Nitta, 1995). These will be large facilities with 130 m^2 of growing area at the Johnson Space Center and 150 m^2 of growing area at the Institute for Environmental Sciences. These facilities are being designed to recycle up to 80% of the solid, liquid and gaseous life support requirements for the enclosed humans and to demonstrate the feasibility of utilizing plants in space bases.

Space technologies

This space-directed programme with plants has led to extensive research efforts in controlled environments to establish the optimal performance of plants for life support. The programme has led to the development of technologies that minimize energy, mass and volume requirements and provide a maximum of reliability and automation in plant growing. Several unique technologies have been developed or exploited for plant growing.

Lighting

One of these technologies is the use of light emitting diodes (LEDs) for providing photosynthetic flux for plants. In the last few years very bright LEDs have been developed, first in the red wavelengths and, more recently, in the blue region that have sufficient intensity to have usefulness for plant growing. LEDs are diodes that provide a specific waveband emission that is characteristic of the compounds used in their construction. The gallium aluminium arsenide diodes provide red LEDs with a waveband commonly peaking at 660 nm and having a 30 nm half-band width (Fig. 1b). These red LEDs can be obtained with peak emissions at intervals between 640 and 940 nm. The gallium nitride LEDs produce a blue light with a waveband peaking at 450 nm and having a 50 nm half-bandwidth (Fig. 1a).

LEDs have advantages for space systems because they have low amounts of longwave radiant emissions, have very long life, and require very little space for mounting. Their electrical/PPF efficiency appears to be nearly as good as high pressure sodium lamps. Wheat plants have been grown successfully under lighting units with only the red LEDs. However, broad-leaved plants require a small proportion of blue LEDs to avoid stem and petiole elongation and obtain normal plant development.

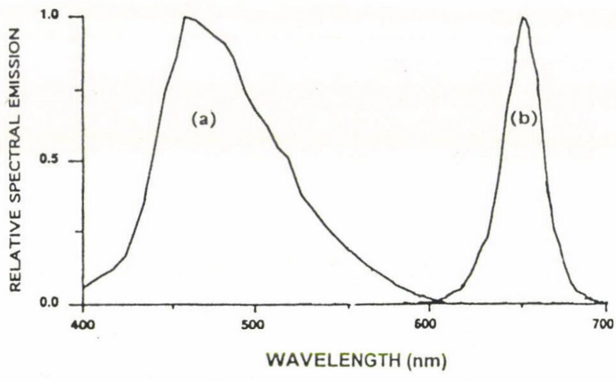

Fig. 1. Spectral photon output of blue gallium nitride (a) and red gallium aluminium arsenide (b) LEDs

Another new lamp type is the microwave lamp. One of the most useful microwave lamps produces radiation by activating sulphur ions, resulting in a broad band spectrum of radiation from 400 to 700 nm with a peak emission at 500 nm (Fig. 2). These lamp units have microwave generators that direct microwave emissions into a small globe-shaped lamp. Screening around the units is necessary to avoid microwave exposure to humans. Initially these lamps were produced in a 3500 watt size and had a rapidly spinning lamp which was cooled by jets of air. They have been evaluated in several laboratories in North America and found effective for plant growing, but require considerable maintenance. Dixon at Guelph, Canada, has fitted two chambers with these lamps and is also evaluating smaller 1000 W lamps providing PPF through light pipes (Dixon et al., 1997). Chamber manufacturers in the United States and Canada are supplying growth chambers with these lamps for plant lighting.

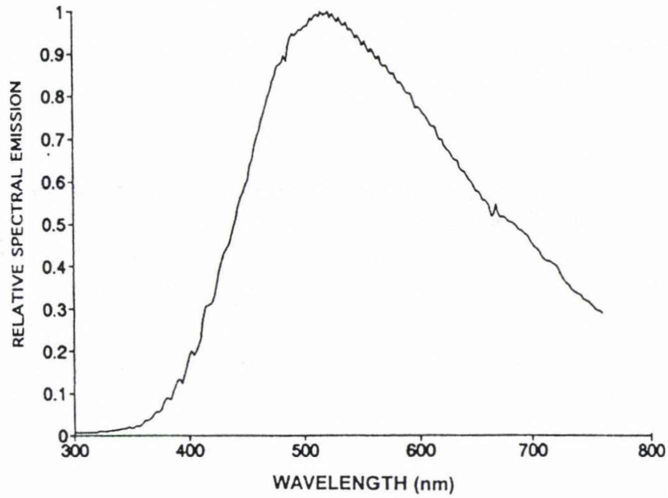

Fig. 2. Spectral photon output of 3500 W sulfur microwave lamp

Watering systems

The difficulties in providing water and nutrients to plants in weightlessness have led to considerable research into the use of porous plates or porous tubes that contain nutrient solution under negative pressures. The solution is provided to the plant roots or to the root media by slow capillary movement through the porous walls and avoids release of any free water. The pores must be less than 50 microns in diameter and the tension is maintained between 0.5 and 1.5 kPa (5 to 15 cm of water tension). A system utilizing stainless steel porous tubes buried in arcillite (baked montmorillinitic clay particles) has been used successfully in shuttle flight experiments by a group at the University of Wisconsin (Duffie et al., 1995; Tibbitts and Frank, 1995). A system utilizing a plastic tube encircled with a slightly larger flexible tube permitting plant roots to grow in the space between the two tubes, is being developed by staff at the Kennedy Space Center (Dreschel and Sager, 1989).

Humidity control

This same technology, using porous tubes and a contained liquid under tension, has been evaluated by the Center for Space Automation and Robotics at the University of Wisconsin for the control of humidity in growth chambers (Morrow and Bula, 1988). The porous tubes are suspended in the chamber and the chamber air directed over the tubes. Through regulation of the temperature of the water within the tubes, the unit can be used to either humidify or dehumidify the chamber air and avoid releasing any free water into the chamber. This system is being utilized in small experiment chambers flown in the space shuttle and as the unit removes water that is transpired by the plants, the water is recycled into the nutrient solution reservoir (Duffie et al., 1995).

Ethylene removal

A serious problem in closed chambers is the accumulation of ethylene. An effective technology for removing ethylene in space systems is the use of photocatalysis for it does not require replacement of a filtering agent at intervals. Units have been constructed with a microporous zirconia-titania catalyst developed by Anderson et al. (1993) and contain a small ultraviolet lamp for irradiating the catalyst (Fig. 3). These have been installed in small plant chambers flown on the shuttle. The response of these units has been studied in detail by Tibbitts et al. (1997), who have documented the importance of keeping these units warm to lower the relative humidity of the contaminated air and maximize ethylene removal. This photocatalytic system is effective in photocatalysing nearly all volatile hydrocarbons that are present in air.

Controlled environment concerns

As the science of phytotronics has evolved many problems unique to growing plants in enclosed environments have been recognized and many have been effectively solved. However, certain problems have not been effectively resolved. I would emphasize a few of these.

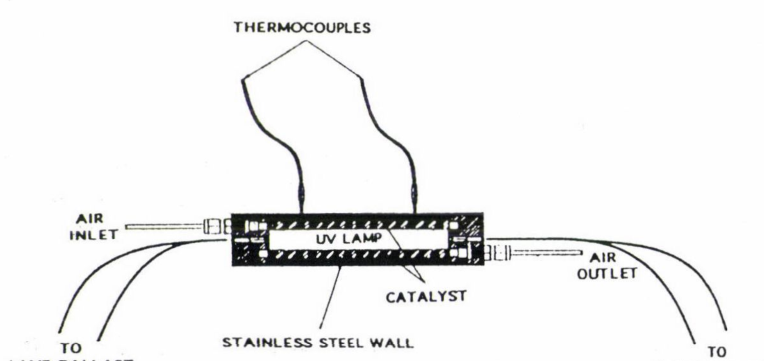

Fig. 3. Diagram of photocatalytic unit

Longwave radiation

A problem that has been of concern for many years is the excessive longwave radiation produced by most lamps. The common growth chamber lamps, fluorescent and high pressure discharge lamps, produce approximately twice as much longwave radiation as found in sunlight outside. Thus, when the PPF exceeds 500 μmol m^{-2} s^{-1}, this longwave radiation becomes quite intense and must be reduced with some type of barrier if excessive plant and soil warming is to be avoided. Transparent acrylic plastic barriers are effective if cooled air is passed over the plastic to keep its surface cool. In some facilities, water barriers are utilized but this involves large installation and maintenance costs (Warrington, 1995). Davis has developed water-jacketed lamps that are very effective in removing longwave radiation from the emissions of high intensity discharge lamps (Davis, 1995). These jacketed lamp units also require considerable installation and maintenance costs.

Volatile contaminants

A continuing concern in controlled environments is volatile emissions from plants and from chamber components that cause toxicity to plants. This is an increasingly serious problem in tightly closed chambers where there is little exchange of chamber air with the ambient air. In the closed chamber at the Kennedy Space Center, 150 different volatile organic compounds were found, and of those identified, 40% were shown to be of plant or microorganism origin and 60% from the materials used in the construction of the chambers (Battan et al., 1996). Thus, toxicity problems are difficult to associate with any particular volatile compound, and this is complicated by the fact that the symptomology and sensitivity of different plant species varies widely. The phytotoxicity of most contaminants is not known. The few that have been identified with toxicity in low ppb concentrations are: a) dibutyl phthalate, a plasticizer used in different types of plastic tubing, sheets and glazing strips; b) ethylene glycol, a commonly used coolant; c) cyclohelalamines used in caulking materials and in fungicides added to steam; d) mercury vapours following breakage of thermometers; e) ethylene released from actively growing plants (Tibbitts 1995, 1997).

Chamber leakage

As attempts are made to develop closed systems, the problems of leakage provide significant problems (Wheeler et al., 1991). Insertions into the closed chambers for power supply, coolant requirements, environmental monitoring and sampling must be very carefully sealed. This careful sealing is needed because of the significant variations in pressure across cooling coils and fans and also because of air expansion and contraction with programmed changes in temperatures. Thus, the 'closed' chambers being utilized today have leakage rates rarely below 2% of the volume per day, and commonly closer to 10% per day.

Future challenges

Phytotrons have several major functions in the ensuing decades and I would like to stress three of these in this concluding section: the study of environmental degradation, indoor contaminants, and the controlled environment production of crops.

Environmental degradation

The need for phytotronics in the study of the environmental degradation of our Earth's biosphere is being exploited today as researchers investigate the impact of projected carbon dioxide increases, potential temperature rise and increasing ultraviolet radiation. Quite certainly, as our world-wide population continues to increase, these and other insults to our environment will become of greater concern for the maintenance of a healthy and life-sustaining biosphere. Controlled environments provide the capability to simulate future levels of changes and thus predict the future impact on the environment, for the development of control strategies and the establishment of regulatory policies.

Indoor contaminants

Indoor contaminants are slowly emerging as a significant concern to humans and to plants. This concern is increasing with the increasing efforts to insulate and seal homes for energy conservation that reduces the amount of fresh air exchange in the indoor environments. The use of controlled environments is a necessity in order that individual contaminants can be isolated and identified and their toxicity established.

Controlled environment production of crops

The production of crops will quite certainly be of increasing importance in the ensuing decades. The likely emphasis in the near future will be on specialty plants, such as high quality salad crops, herbs, fruits, etc. that can command high prices through insuring a uniform product of high quality. Continuing effort will be needed to develop procedures that can automate the production and minimize the chances for destructive cultural problems that will significantly alter a crop's quality or productivity.

In emphasizing the future challenges for phytotronics, one cannot discount the continuing role that phytotrons will play in helping to understand the complex interacting roles of the separate environmental conditions controlling the growth of different plant species. Phytotrons are particularly needed in regions where electrical power cannot be depended upon for uninterrupted service, and thus where central facilities with back-up power generation capability are a necessary cost-effective solution. The challenges in phytotronics are very large and exciting. Phytotron facilities are needed and I believe they will continue to be centres of research activity around the world for several more decades.

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FACTS AND FIGURES ON THE MARTONVÁSÁR PHYTOTRON

T. TISCHNER

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462, MARTONVÁSÁR, P.O. BOX 19, HUNGARY

The fifty or so climatic plant growth and testing units in the phytotron opened in the Agricultural Research Institute of the Hungarian Academy of Sciences on November 3rd 1972 provided the technical conditions required for exact plant research in Martonvásár. In the high quality phytotron units, manufactured by the Canadian company Controlled Environments Ltd. (Conviron) the climate of any part of the world where vegetation exists can be simulated in reproducible programmes, with temperatures ranging from –25°C to +45°C. Over the past quarter of a century a total of 2981 experiments have been carried out. In addition to developments in the field of artificial illumination, the most significant technical innovation achieved in the Martonvásár phytotron was the development of the gradient plant growth chamber, in which gradients, perpendicular to each other, can be created on the growth area for two chosen environmental factors. The first 25 years of the Martonvásár phytotron are hallmarked by 320 publications relating to the experiments set up in the growth units.

Key words: controlled environment, cross-gradient bench, phytotron, plant growth chamber

Introduction

Plant biologists have long been interested in how the major environmental factors required for the growth and development of experimental plants, such as temperature, light, air humidity, etc., could be created at any time of the year in a programmable, reproducible manner independently of the weather. By the middle of the century refrigeration, lighting and electronic regulation techniques had reached a standard which made it possible to manufacture plant growth equipment capable of satisfying these criteria. The plant research facilities containing these climatic units were named phytotron (from the Greek words phyto = plant and tron = house) at the suggestion of the head of the first phytotron built in Pasadena, USA in 1949 (Went, 1950).

The idea of establishing a phytotron in Martonvásár was born in 1959 at a scientific conference organized to celebrate the 10th anniversary of the foundation of the institute. Exact reproducibility of experimental conditions was an essential precondition of the plant genetic research conducted at the institute. Naturally, it was also clear that the experimental facilities set up in the phytotron would be important in other i.e. plant physiological, cell biological and breeding research.

The Presidium of the Hungarian Academy of Sciences discussed already in 1961 the idea of setting up the Martonvásár phytotron and came to a decision that "an up-to-date phytotron should be constructed as soon as possible in order to further improve the standard of biological basic research and plant breeding research, being conducted at the institute".

The actual planning and building work could only begin in 1970. This was when the agreement with the Canadian company Controlled Environments Ltd. (Conviron) was signed, too, for the manufacturing and shipping of 44 plant growth chambers. In the second year of the building work, not only the plant growth chambers were installed, but trial experiments were set up, too. The official opening of the phytotron took place on November 3, 1972.

Technical parameters

The 30 m × 30 m area which houses the phytotron units is situated in the centre of the two storey building, which has a total ground area of 50 m × 50 m (Fig. 1). In the phytotron units, manufactured by the Conviron company, the climate of any part of the world which is covered with vegetation can be simulated, programmably and reproducibly, from –25°C to +45°C. Modern computerised techniques have allowed the elaboration of up-to-date research technologies not only in the field of programming, regulation and data collection, but also for the processing and analysis of experimental results. The major technical parameters of the plant growth and testing equipment are presented in Table 1 (Tischner et al., 1997).

The central hall is surrounded by up-to-date laboratories, work rooms, processing rooms and offices for the researchers.

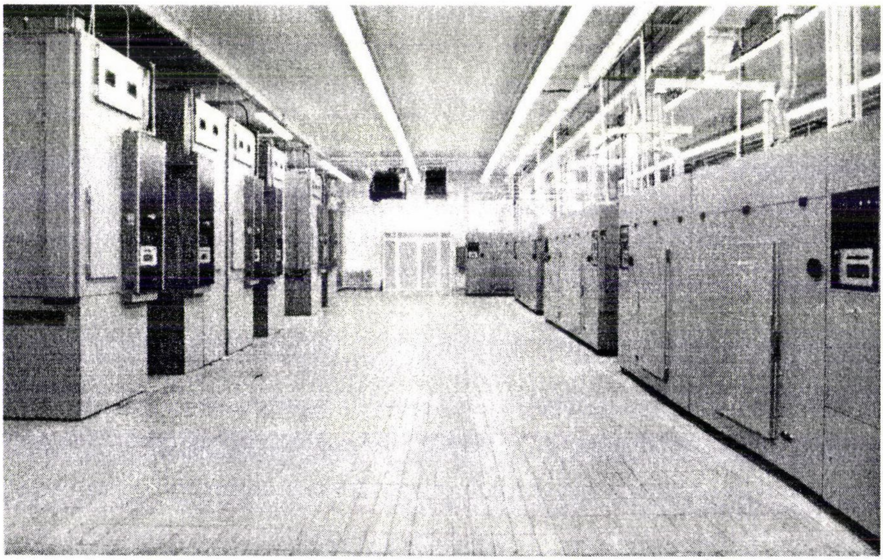

Fig. 1. The phytotron hall

Table 1
Technical parameters of the phytotron units

Phytotron unit			Growth		PPFD*	Temperature	
Designation	Type	No.	area (m^2)	height (m)	max. ($\mu mol/m^2s$)	min. (°C)	max. (°C)
Before reconstruction							
Autumn-winter chamber	PGV-36	12	3.3	2.4	500	–5	40
Spring-summer cabinet	E-15	12	1.4	1.8	500	5	40
Spring-summer cabinet	E-15VH	4	1.4	1.8	1000	5	40
Growth bench	GB-48	14	4.3	2.8	325	15	35
Cold room	C-812	2	7.1	0.5	–	–20	20
Gradient chamber	GRD-01	1	3.3	1.5	400	5	35
Germination cabinet	G-30	5	1.5	0.3	65	4	40
Vernalisation chamber	J-01	1	13.5	0.4	10	2	20
After reconstruction							
Autumn-winter chamber	PGV-36	12	3.3	1.9	600	–10	40
Spring-summer cabinet	PGR-15	16	1.4	1.8	600	4	45
Growth bench	PGB-96	6	9.0	2.6	500	10	40
Growth bench	GB-48	6	4.3	2.8	325	15	35
Cold room	C-812	2	10.8	0.5	–	–25	20
Gradient chamber	GRD-01	1	3.3	1.5	400	5	35
Germination cabinet	G-30	5	1.5	0.3	65	4	40
Vernalisation chamber	J-01	1	13.5	0.4	10	2	20
Tissue culture chamber	TCL	2	25.0	0.3	90	20	30

*Photosynthetic Photon Flux Density

Experiments

Over the last quarter-century 2981 experiments have been carried out in the Martonvásár phytotron. This represents an annual average of 125 experiments. The duration of the experiments averaged 3–4 months, though some were considerably longer or shorter than this. The average annual net utilisation of phytotron capacity ranged from 60–70%. Even during the reconstruction work, carried out in two phases in 1989–1990, research was continued, though with reduced capacity.

The 25 years which have passed since the phytotron was opened represent more than half the life of the institute itself, and during this period numerous research projects have been initiated and discontinued, some research teams have been amalgamated, others have been divided and very few have remained constant throughout in both name and purpose. For this reason it is impossible to provide a detailed analysis here of the phytotron experiments in terms of years and research teams. Table 2 only give a general picture of how the work was distributed between the larger organisational units and research topics.

Table 2
Number of experiments set up for various research groups (1973–1996)

Year	Research topics													
	A.G.	F.B.	H.W.	F.M.	P.R.	R.B.	W.B.	M.B.	M.P.	P.G.	S.R.	P.D.	O.I.	SUM
1973	35	1	4	6	–	–	8	20	–	11	–	1	6	92
1974	32	2	2	–	–	–	21	22	–	4	–	7	11	101
1975	30	–	–	–	–	2	27	15	–	6	–	6	21	107
1976	45	1	1	1	–	1	25	5	–	5	–	9	25	118
1977	29	4	–	9	–	3	39	14	–	5	–	2	21	126
1978	19	6	2	10	–	7	41	14	–	6	–	3	35	143
1979	7	10	2	17	–	10	44	4	–	12	–	1	42	149
1980	13	12	3	16	–	4	46	9	9	4	–	1	45	162
1981	21	–	–	19	–	2	22	–	17	15	3	11	22	132
1982	19	–	–	19	16	9	–	–	8	27	7	8	17	130
1983	–	–	–	20	45	11	12	–	4	38	5	–	17	152
1984	–	–	–	5	24	9	39	14	2	35	3	–	22	153
1985	–	–	–	2	15	31	19	2	3	52	4	–	27	155
1986	–	–	–	–	9	10	16	15	15	55	3	–	21	144
1987	–	–	–	–	12	16	31	9	10	40	3	–	16	137
1988	–	–	–	–	13	15	28	–	13	39	3	–	8	119
1989	–	–	–	–	5	11	22	3	14	22	–	–	11	88
1990	–	–	–	–	10	13	13	6	6	31	–	–	12	91
1991	–	–	–	7	27	23	18	4	4	32	–	–	3	118
1992	–	–	–	–	35	11	9	3	1	17	–	–	11	87
1993	–	–	–	–	26	14	44	1	1	18	5	–	5	114
1994	–	–	–	–	42	12	33	1	–	13	7	–	12	120
1995	–	–	–	–	–	34	37	2	2	32	7	2	8	124
1996	–	–	–	–	–	17	42	2	4	38	4	1	11	119
SUM	250	36	14	131	279	265	636	165	113	557	54	52	429	2981

Abbreviations: A.G.: Autumnisation Genetics, F.B.: Flowering Biology, H.W.: Hybrid Wheat, F.M.: Frost–test Methodic, P.R.: Physiology Research, R.B.: Reproduction Biology, W.B.: Wheat Breeding, M.B.: Maize Breeding, M.P.: Maize Production, P.G.: Plant Genetics, S.R.: Seed Research, P.D.: Phytotron Development, O.I.: Other Institutions

Right from the beginning experiments have also been set up for other institutes. The 429 experiments carried out for external clients made up 15 % of the total number of experiments. Of the over 40 institutes and companies involved, mention should first be made of the National Institute for Agricultural Quality Control, for whom the frost resistance of winter wheat and barley varieties and experimental lines has been tested each year since 1976 as part of the state variety trials. Experiments have also been set up for other institutes on several dozen other plants, including chamomile, hemp, rape, periwinkle, oyster mushrooms, tomatoes, soybeans, carnations, etc. In recent years the companies AGROTAB and CEREOL have been our most faithful partners, ordering breeding experiments on tobacco and sunflower.

Results

The plant growth and testing units in the phytotron are research tools in which the conditions required for exact plant research can be created. When plant experiments in phytotron units are concluded, it can rarely be said that the problem in question has been completely solved. In many cases further phytotronic experiments are required. Very often the phytotron experiments are preceded or followed by field trials. In this way the phytotron cooperates indirectly in the creation of new varieties, in the elaboration of new agronomic techniques and in the solution of basic research problems in physiology and genetics. Enumerating the results, the methodological research (i.e. climatic program elaboration) and the technical development constantly present in the phytotron throughout the past 25 years should be mentioned. The results of phytotronic research can only be numerified as the number of publications related to the experiments.

Results in methodological research

The climatic programmes used in the plant growth equipment, or phytotron units, are fundamentally determined by the aims and strategy of the research. The research strategy in the Martonvásár phytotron was elaborated by Rajki (1973). This research strategy aimed to simulate natural conditions in the climatic programmes used for experiments. A method for calculating phytotronic climatic programmes by fitting trigonometric functions to climatic data series measured by meteorologists was elaborated in Martonvásár by Pletser (1973). Naturally, when preparing any specific climatic programme, the technical parameters of the given phytotron unit must also be taken into consideration.

Apart from the phytotronic climatic programmes which simulate natural climatic conditions, special, extremely simplified programmes are also used in many cases. In these programmes the simplification is frequently possible because of the nature of the experiment (e.g. the constant +2°C temperature and daily 8 h illumination at 1 klx in the vernalisation cabinet), while in other cases the simplification is necessary because of the very simple regulating system of the given phytotron unit.

During the 25 years since the Martonvásár phytotron went into operation a large number of climatic programmes have been elaborated and utilised. The most frequently used climatic programmes are published by Tischner et al. (1997).

Results in technical development

In addition to development work on the artificial illumination of plants (the optimisation of fluorescent lamp combinations, the development of light canopy with daylight metal halide lamps, etc.), the most important technical advance achieved in the Martonvásár phytotron was the construction of a gradient plant growth chamber. This gradient chamber, patented by a team of private and institute inventors, was conceived as the further development of an idea used in metallurgical analysis and is excellent for the solution of optimisation problems. The first experiments were set up in autumn 1973 and a patent was applied for first in spring 1974 and again, due to new problems which arose in the meantime, in autumn 1975. The cross-gradient chamber was finally patented in Canada, Germany, Hungary, Japan and the USA (Horváth et al., 1978).

With this equipment it is possible to create perpendicular gradients on the growth surface for two selected environmental factors. While hundred pairs of values are created in a regular arrangement for the two selected factors, the other factors remain constant for all the experimental plants. If the gradient values are wisely chosen a single experiment may be sufficient to demonstrate the optimum values of the two environmental factors, thus saving substantial quantities of energy, time and experimental material. From the methodological point of view, it has the further advantage that the duration of the gradient effects can also be programmed as a third factor (Tischner and Veisz, 1996).

Publications

The 320 publications (22 books and book chapters, 23 dissertations, 178 papers in scientific journals, 61 conference proceedings and 36 articles in non-scientific publications), the results achieved with the indirect assistance of the experiments, such as new varieties, plant protection and agronomic techniques, etc., and the gradient plant growth chamber, designed as the result of technical development, all hallmark the 25 hard-working years of the Martonvásár phytotron.

Epilogue

On November 3rd, 1972 the official opening of the phytotron took palce as a part of a scientific conference (Colloquium on Winter Hardiness of Cereals). Following the good example, the celebration of the 25th anniversary of the opening was also connected to an international scientific symposium (International Symposium on Cereal Adaptation to Low Temperature Stress in Controlled Environments), where with the presentation of 40 papers and 25 posters, the scientists arriving from 20 countries, proved the indispensability of phytotronics in plant research.

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ORIGINAL PAPERS

Acta Agronomica Hungarica, Vol. 45(4), pp.361–370 (1997)

ACCLIMATION AND TOLERANCE TO CHILLING AND FREEZING STRESS: THE INVOLVEMENT OF PLANT HORMONES

K. DÖRFFLING, M. ABROMEIT, B. CAPELL and F. JANOWIAK*

INSTITUTE OF GENERAL BOTANY, UNIVERSITY OF HAMBURG, OHNHORSTSTRASSE 18, D–22609 HAMBURG, GERMANY
*INSTITUTE OF PLANT PHYSIOLOGY, POLISH ACADEMY OF SCIENCES, PODLUZNA 3, PL-30–239 CRACOW, POLAND

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The processes which are induced in plants by low temperatures and which lead to cold acclimation are only partially understood. The possible involvement of plant hormones is discussed controversially in the literature and has obviously been insufficiently investigated. The present paper describes changes in the content of plant hormones (ABA, t-ZR, ACC, MACC) and their possible function in the acclimation process to chilling temperatures in maize and to freezing temperatures in winter wheat.

Key words: abscisic acid, acclimation, chilling, cytokinins, freezing, *Triticum aestivum, Zea mays*

Abbreviations: ABA – abscisic acid, ACC – 1-aminocyclopropane-1-carboxylic acid, MACC – 1-(malonylamino)-cyclopropane-1 carboxylic acid, t-ZR – trans-zeatinriboside

Introduction

Plant life is greatly affected by low temperature stress. Maize, a plant species of tropical origin and of great economic importance, does not survive subzero temperatures and is affected greatly by chilling temperatures above zero. Chilling tolerance within this species varies considerably between genotypes, as does the ability to acclimate to chilling temperatures. This makes maize a suitable model plant for studies of the acclimation processes to chilling temperatures and the chilling stress phenomena as well.

Winter wheat, on the other hand, is a species which can tolerate freezing temperatures. As in maize, genotype-specific differences in acclimation ability as well as in freezing tolerance exist in winter wheat. Therefore, and because of the economic importance of this crop species, great efforts have been made to explore the physiological, biochemical and molecular processes of acclimation to freezing stress in winter wheat and related cereals, but nevertheless the mechanisms involved are only partially understood (Sakai and Larcher, 1987; Dörffling et al., 1994).

Controversial opinions exist about the involvement of plant hormones in cold acclimation and cold stress phenomena. Transient increases in abscisic acid (ABA) levels have been observed in several investigations as an early response to cold hardening temperature in freezing-tolerant species like wheat (for literature see Cowan et al., 1997), but are denied by other authors (Dallaire et al., 1994). Chilling-sensitive species including maize have also been found to exhibit increased levels of ABA when exposed to low temperature (Daie et al., 1981; Capell and Dörffling, 1989; 1993; Janowiak and Dörffling, 1996a). The question whether these transient or long-lasting increases are caused directly by the lowered temperature or indirectly by low temperature-induced water deficit has not been answered clearly, nor has the question whether the observed hormonal changes have an adaptive value.

With regard to cytokinins, Taylor et al. (1989) observed changes opposite to those of ABA during cold hardening and during a whole winter period in leaves and crowns of winter wheat. Whereas ABA levels in field-grown Norstar wheat plants rose during autumn and early winter, reaching a maximum in December, the total cytokinin level decreased to minimum amounts between December and March and rose again afterwards. The authors proposed a role for ABA in the acclimation process and for the cytokinins in the process of dehardening. Decreasing cytokinin levels have been observed also when *Euphorbia pulcherrima* plants were chilled (Tantau and Dörffling, 1991).

Changes in ethylene, its precursor ACC and its conjugate MACC have been studied in winter wheat cultivars of different frost tolerance by Machackova et al. (1989). They stated a sharp decrease in ethylene formation when the temperature was lowered, whereas ACC production reacted in the opposite direction. Genotypic differences in the production of these two plant growth regulators were not observed, but the level of MACC, which rose rapidly during the first days of cold, was positively correlated to freezing tolerance.

In the following chapters we review our own recent investigations on changes in ABA, the cytokinin t-ZR as well as the ethylene precursor ACC and its conjugate MACC during low temperature treatment of maize in relation to other physiological parameters. Furthermore, we report on studies on the function of ABA during cold hardening in winter wheat.

Materials and methods

Studies with maize were performed with seedlings of more than ten genotypes with clearly defined differences in their chilling tolerance on the basis of measurements of necrotic injuries and electrolyte leakage. ABA and t-ZR levels were measured by immunological assays mostly in the third leaf of seedlings which were in the 3–4 leaf stage. ACC and MACC levels were measured by gas chromatography after chemical conversion to ethylene. Growth chamber - cultivated (at 24/22°C, day/night) plants were chilled at 5/3°C with and without previous acclimation at 14/12°C. In some cases only the roots were chilled. The methods are described in detail in publications of Capell and Dörffling (1993) and Janowiak and Dörffling (1995; 1996a,b). Changes in ABA levels in field-grown maize plants were measured during and after occasional cold air waves in May and June at two locations, Cracow and Hamburg.

Several genotypes of winter wheat with differences in their frost tolerance, as determined by different test methods including field winter survival, served for studies on the role of ABA in the cold acclimation of this species. They were either grown and cold-hardened in darkness or under light conditions in growth chambers and in the field. Details are described in Dörffling et al. (1990), Abromeit et al. (1992) and Abromeit and Dörffling (1994).

Results and discussion

Changes in ABA, t-ZR, ACC and MACC in maize in response to chilling

The idea that ABA may be involved in the chilling tolerance of maize results from two observations. First, survival of chilling can be considerably improved by the application of ABA and/or synthetic ABA analogues to young seedlings prior to the exposure to low temperature (Flores et al., 1988). Second, chilling stress is, at least in some cases, "secondary water stress", which means that chilling lowers the leaf and shoot water potential, mainly by reduction of hydraulic conductivity and by "locking open" of the stomata. A role of ABA in water stress is generally accepted. The decrease in water potential in the genotypes studied was found to be correlated negatively with the genotype-specific chilling tolerance. This means that chilling-tolerant genotypes suffer under smaller water deficits and smaller decreases in water potential than chilling-sensitive genotypes (Capell and Dörffling, 1993; Janowiak and Dörffling, 1996a). These observations are in line with the additional finding that chilling temperatures are more injurious when the ambient humidity is low, so that water deficit develops more severely. Vice versa, high humidity protects maize and other chilling-sensitive species against chilling temperature (Janowiak and Dörffling, 1996a).

Theoretically several approaches are possible to studying the involvement of ABA in chilling and freezing tolerance: the use of ABA-deficient mutants, of ABA biosynthesis inhibitors, or simply by comparing ABA relationships in genotypes which differ in chilling tolerance. The first approach was not possible because the necessary mutants are not available. The second approach is promising, but has not yet been used in our studies with maize (but in those with wheat reported below). Anderson et al. (1994) have already shown that the ABA biosynthesis inhibitor fluridone suppressed the ability of acclimated maize seedlings to tolerate chilling. The third approach, which we are using, provides correlative data between ABA levels and the development of chilling tolerance and chilling injury. Such studies by themselves are not suitable for the establishment of causal relationships, but together with other data they may nevertheless give valuable information about the chain of events which occurs during the acclimation and chilling process.

Contrary to some other reports we found significant ability of the chilling-tolerant as well as the chilling-sensitive maize genotypes to improve their chilling tolerance (to 5/3°C) by an acclimation phase at 14/12°C for four days which followed the growth phase at 24/22°C. During the acclimation phase no changes in the level of ABA in the third leaves occurred in the genotypes tested. However, during exposure to chilling (5/3°C for 5 days) the level of ABA rose depending on the genotype and the ambient humidity. The increase was up to fivefold in chilling-tolerant genotypes at 65 to 70% humidity, but only two- to threefold in sensitive genotypes. High humidity (100%) reduced the increase in ABA (and the severity of chilling-induced stress phenomena, as stated above).

A preceding acclimation phase had a double effect on the ABA status during the subsequent chilling period: the increase in ABA was faster and greater in acclimated than in non-acclimated seedlings. Moreover, a clear difference between tolerant and sensitive genotypes became evident: the threshold water potential, at which the rise in the ABA level during chilling began, was much higher (less negative) in tolerant than in sensitive genotypes. Thus, not only the higher level of ABA, but also the earlier production (in time and in terms of water potential decrease) of more ABA may reflect a better protection mechanism against chilling stress in the tolerant genotypes.

This relationship has been confirmed in principal for 14 maize genotypes (Capell and Dörffling, 1993; Janowiak and Dörffling, 1996a), so that it obviously reflects a general character, at least in this species and at least under the experimental conditions described. Similar observations have been made with regard to drought stress in maize (Quarrie, 1991). A drought-tolerant genotype was found to produce higher levels of ABA during a drought period and to start additional ABA production at a less negative water potential than a drought-sensitive one.

However, it is not clear whether the chilling-induced increase in ABA in maize is solely determined by the water status. Several lines of evidence point to a direct effect of the lowered temperature on the ABA synthesizing system. For example, Janowiak and Dörffling (1996a) found that acclimated seedlings of a chilling-tolerant line did not change their water content during chilling at 100% humidity, but their ABA content rose significantly. Moreover, recent studies on maize seedlings during cold periods in the field in spring at two locations (Cracow and Hamburg) confirmed the higher ABA accumulation in the tolerant genotypes than in the sensitive ones (Fig. 1), but a water deficit was not observed in both groups. An independence of the ABA content from changes in water relations has also been suggested by Daie et al. (1981), who observed ABA accumulation in chilled plants of various species in the absence of water deficit. Zhang et al. (1986) also concluded that the ABA content under chilling conditions is probably directly affected by the temperature, and not by chilling-induced water deficit. Obviously further research is needed here.

The accumulation of ABA during chilling may protect the plant by several mechanisms. Closure of stomata and the ensuing reduction in water loss is probably one of them. A general association of higher diffusive resistances with higher ABA levels in the chilling-tolerant genotypes (Capell and Dörffling, 1993) provides evidence for this. A further mechanism may be the enhancement of root hydraulic conductivity (Ludewig et al., 1988). The observation that ABA improves chilling tolerance in suspension-cultured cells points to a third mechanism at the cell level (Xin and Li, 1992).

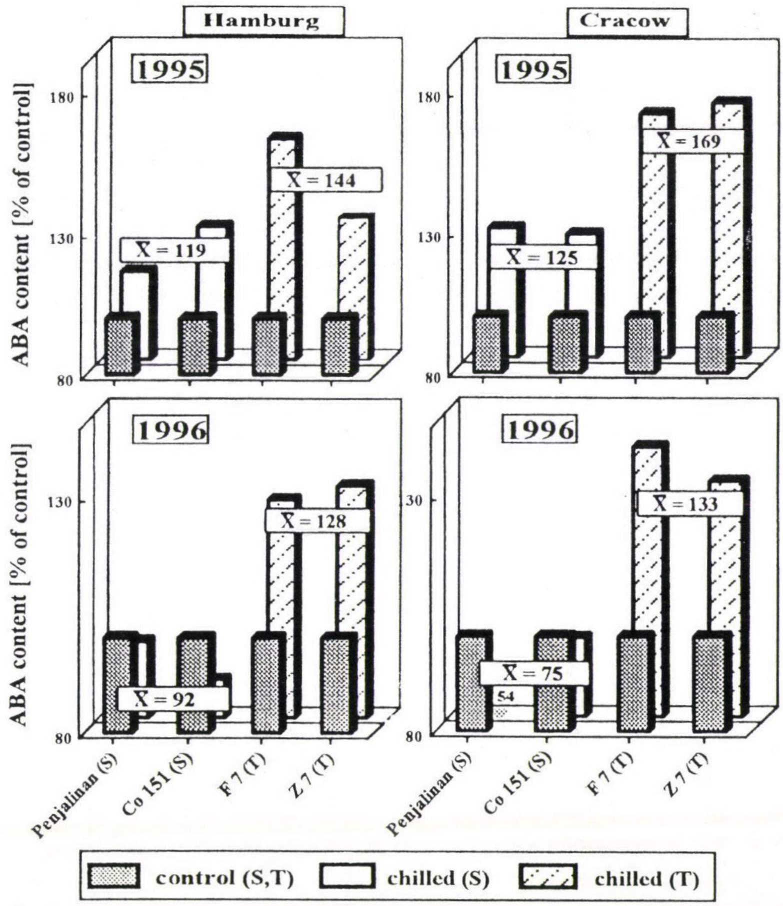

Fig. 1. Relative ABA content in the third leaves of maize seedlings under field conditions during normal (control) and chilling weather conditions in the years 1995 and 1996 at two sites of Europe (Hamburg and Cracow). (S) chilling-sensitive genotype, (T) chilling-tolerant genotype.

The finding that chilling-tolerant genotypes of maize are characterized by the faster and greater accumulation of ABA as a chilling-protective hormone may serve as a basis for improving chilling-tolerance in this species by selection and conventional breeding for "high-ABA plants". To our knowledge this has not been tried until now. However, with regard to the related stress phenomenon drought tolerance such an approach has been started with promising results (Quarrie, 1991).

In comparison to the reported studies on ABA, much less information is at present available on changes in cytokinin levels in chilled maize plants. The available data point to a similar reaction as in the studies with wheat mentioned above. When the roots of a chilling-tolerant inbred line (F7) were chilled, the t-ZR content of its leaves decreased to a very low level, whereas no change was observed in a chilling-sensitive inbred line (Co 151) (Janowiak and Dörffling, 1997). Since the ABA levels rose under this treatment, the ABA/t-ZR ratio changed greatly and differed drastically between the two inbred lines. This suggests that cytokinins in addition to ABA may have a function in the acclimation process to chilling, but much more experimental data is necessary to support this hypothesis.

This statement is valid also when changes in ACC and MACC contents are considered. Chilling caused an increase in ACC levels, which were significantly higher in chilling-sensitive than in tolerant maize genotypes (Janowiak and Dörffling, 1995; 1996b). The MACC levels rose also, but did not differ significantly between the genotypes. These data do not indicate any adaptive value of the changes in these growth regulators. However, ACC levels like cytokinin and ABA levels may at least serve as biochemical indicators for chilling tolerance in this species.

Studies on winter wheat: changes in ABA during cold treatment, effects of norflurazone

Chilling and freezing stress have some common features, but differ also distinctly. A common phenomenon in both stresses is the involvement of water stress. In chilled plants water stress is caused by reduced water supplies from the roots and by excessive water loss through the stomatal pores, which are unable to close. Freezing stress is accompanied by the formation of ice in the apoplastic space, which withdraws water from the unfrozen cells.

Acclimation to freezing temperatures occurs in winter wheat in at least two phases. In the first phase, hardening temperatures just above zero lead to moderate, genotype-specific frost tolerance, which reaches an optimum after about four weeks. In a second phase subzero temperatures further increase the frost tolerance. The first phase is a prerequisite of the second. Under field conditions the two phases may overlap.

Several lines of experimental evidence support our hypothesis that ABA is involved in the process of cold acclimation in this species:

- Exogenous ABA substitutes partially for the effect of the hardening temperature in cold acclimation. This has been shown in many investigations including a recent one by the present authors (Abromeit et al., 1992);
- Soon after the onset of low temperature conditions in the field or in growth chambers a transient increase in the ABA level can be observed in plants and even calli, which is positively correlated with the genotype-specific frost tolerance (Fig. 2). This transient increase in ABA occurs before a significant increase in frost tolerance can be observed.
- Application of the ABA biosynthesis inhibitor norflurazone to dark-grown etiolated seedlings (which can be cold hardened like light-grown green seedlings) completely abolishes the transient increase in ABA during the first

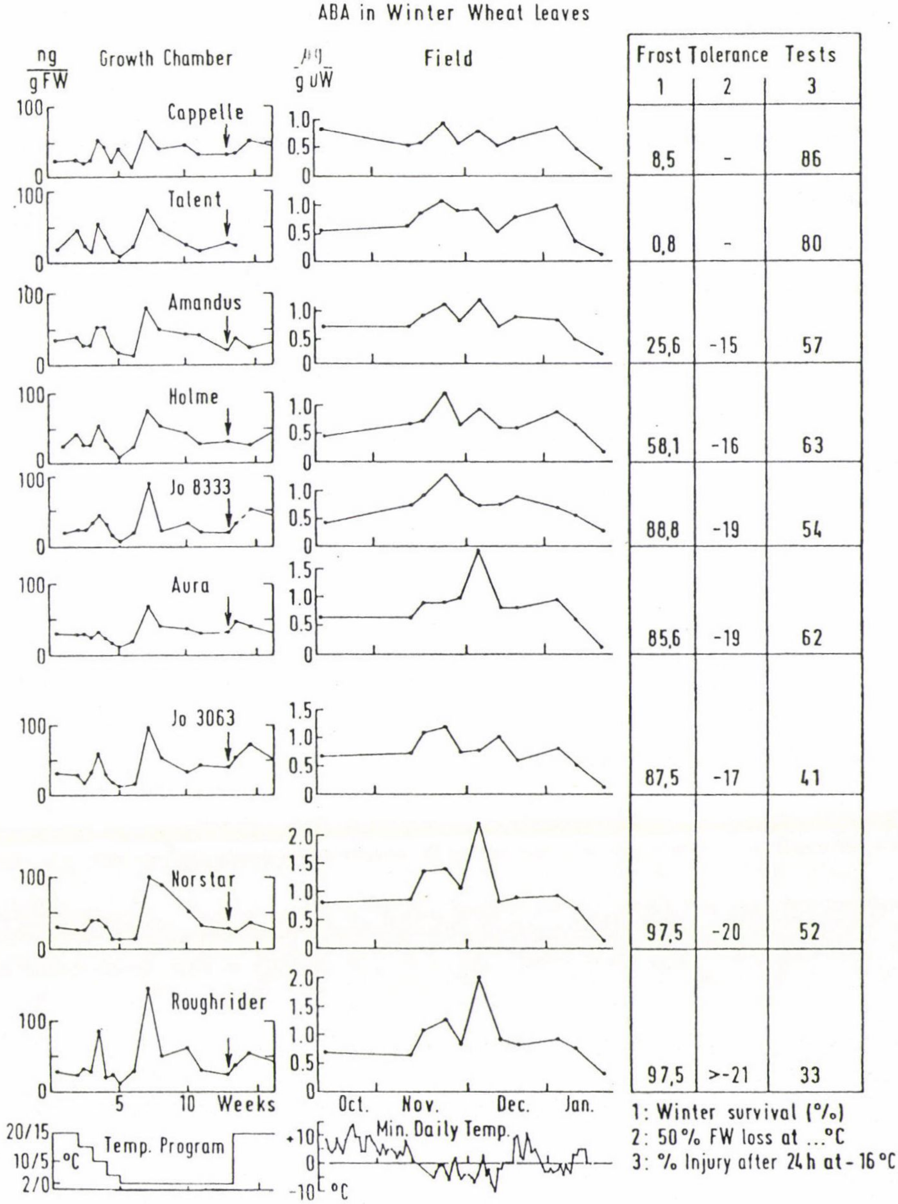

Fig. 2. Changes in leaf ABA content in nine winter wheat genotypes under cold-hardening conditions in a growth chamber and in a field near Hamburg, Germany. The temperature conditions are indicated below. Three different frost tolerance tests were used. Their results indicate the genotype-specific differences in frost tolerance which increases from cv. Cappelle to cv. Roughrider. The winter survival was tested in North Dakota, USA. From Dörffling et al., 1990, modified

phase of hardening and reduces the development of frost tolerance significantly (Abromeit and Dörffling, 1994). Moreover, norflurazone prevents the formation of a group of high-molecular weight proteins (200 kDa), which are closely related to the development of frost tolerance and can be induced by low temperature as well as by application of ABA. However, the fact that the development of frost tolerance in these experiments could not be completely suppressed by norflurazone, must be taken as evidence that ABA is not the only primary factor in the signal transduction chain of cold hardening.

The exact function of ABA in the process of cold acclimation in wheat has not been clarified by these studies. Obviously the increase in the ABA level is a fairly early response of the plant cells to low temperature. Whether low temperature triggers the increase in ABA directly or indirectly (by cold-induced loss of cell turgor?) remains to be investigated. The increased influx of calcium in response to low temperature observed by some workers may be a result or even a cause of the change in ABA. Several ABA-dependent processes have been described, for example the above-mentioned formation of high-molecular weight proteins, which are induced by ABA via gene activation (Sarnighausen and Dörffling, 1994). Changes in phospholipids and sterols may also be involved (Farkas et al., 1985). Moreover, ABA may trigger the formation of compatible solutes like proline, which obviously contributes to the development of frost tolerance (Dörffling et al., 1997).

Concluding remarks

Similarities and differences between the two investigated plant species, which represent a chilling- and frost-sensitive (maize) and a chilling- and frost-tolerant plant type (wheat), with regard to their ABA status are evident. In both species low temperature caused an increase in the ABA level, which was found to be positively correlated with their chilling and freezing tolerance, respectively. Circumstantial evidence was provided that the increased ABA level somehow improves low temperature tolerance. However, the tolerance range under the control of ABA seems to be small in comparison to the great genetical differences in low temperature tolerance between the species. Nevertheless, manipulation of the ABA status, either by selection of "high-ABA plants" in both species or by molecular breeding, may be a suitable tool in the attempt to improve the low temperature tolerance of these important crops.

In general, similarities between the two species are also evident when changes in cytokinins, ACC and MACC are considered. In both species low temperature causes a decrease in the cytokinin level (Taylor et al., 1989, and this paper) and an increase in ACC and MACC (Machackova et al., 1989, and this paper). Although the available data are not sufficient to formulate solid conclusions, the working hypothesis may be allowed that the parallel hormonal changes reflect similar reactions and adaptations to low temperature stress. More investigations are necessary, not only with regard to the growth regulators mentioned, but also to other hormones, for example gibberellins and auxins.

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PHOTOSYNTHETIC CARBON METABOLISM IN LEAVES OF COLD-HARDENED AND NON-HARDENED WINTER RYE AT DIFFERENT AMBIENT AIR TEMPERATURES

O. KEERBERG[1], P. GARDESTRÖM[2], H. IVANOVA[1], H. KEERBERG[1], P. TALTS[1] and T. PÄRNIK[1]

[1]INSTITUTE OF EXPERIMENTAL BIOLOGY, EE3051 HARKU, ESTONIA
[2]DEPARTMENT OF PLANT PHYSIOLOGY, UNIVERSITY OF UMEA
S-901 87 UMEA, SWEDEN

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Leaves of cold-hardened (CH, grown at 5°C) and non-hardened (NH, grown at 25°C) winter rye (*Secale cereale* L.) were exposed to $^{14}CO_2$ under saturating light at 25°C or 5°C and the kinetics of ^{14}C incorporation into the products of steady-state photosynthesis was determined. From the kinetic data the rates of carbon fluxes and the pool sizes of metabolites in the CO_2 assimilation system were calculated. Lowering the temperature from 25°C to 5°C did not change the rate of photosynthesis in CH leaves while in NH leaves a 30% decrease was detected. At both temperatures the ratio of pools of ribulose bisphosphate to hexose monophosphates was higher in CH leaves, indicating that cold hardening results in an increase in the enzymatic capacity of the regenerative phase of the reductive pentose phosphate cycle. The ratio of the serine/glycine contents was significantly higher in CH rye, suggesting an increased activity of the glycine decarboxylase system in cold-hardened plants. Acclimation to the low growth temperatures resulted in the severe suppression of starch synthesis and an increase in carbon incorporation into the intermediates of the respiratory pathways. The rates of sucrose synthesis were equal in NH and CH rye at both measurement temperatures.

Key words: winter rye, *Secale cereale* L., photosynthetic carbon metabolism, cold hardening, leaf temperature

Introduction

Acclimation of plants during growth at low temperatures leads to substantial changes in the photosynthetic carbon metabolism. It has been shown that cold hardening results in an increase in the photosynthetic capacity, measured as the rate of O_2 evolution at saturating levels of CO_2 and irradiance (Öquist et al., 1993). This was accompanied by a decrease in sensitivity to photoinhibition (Öquist and Huner, 1993) and an increase in the activity of the key enzymes of sucrose synthesis and of the reductive pentose phosphate cycle (Hurry et al., 1995). An increase in the content of soluble sugars with a specific accumulation of fructans has been demonstrated in response to cold acclimation (Chatterton et al., 1988; Livingston et al., 1989). These data indicate that the reaction system of the photosynthetic carbon metabolism must have been rearranged during cold acclimation. However, no quantitative information about the changes in the rates of partial reactions in the biochemical system of CO_2 assimilation is available so far. The goal of this study was to establish (1) how the rates of carbon fluxes and pool sizes of the intermediates of this system are modified during cold acclimation, and (2) how the photosynthetic carbon metabolism in leaves of cold-hardened (CH) and non-hardened (NH) plants responds to changes in the ambient air temperature. To this end we analysed the kinetics of ^{14}C incorporation into the products of steady-state photosynthesis in leaves of CH and NH winter rye exposed to $^{14}CO_2$ at 5°C and 25°C.

Materials and methods

Winter rye (*Secale cereale* L., cv. Musketeer) was grown in vermiculite under fluorescent lamps at 25°C (NH plants) or at 5°C (CH plants). Fully expanded third or fourth leaves of NH and CH plants at the same stage of development were exposed to $^{14}CO_2$ (300 μmol mol^{-1}) for different time intervals ranging from 5 s to 10 min. Exposures were performed at 25°C or 5°C under saturating light. After the exposure the leaves were killed in liquid nitrogen. Labelled photosynthates were extracted with cold perchloric acid and separated by paper chromatography combined with the additional separation of phosphorylated compounds in a Partisil column (5×150 mm) and amino acids using an AAA 339 analyser. The radioactivity of individual compounds was determined and plotted against the duration of exposure to $^{14}CO_2$. The curves obtained were analysed according to a special interpretation procedure enabling us to calculate the rates of carbon fluxes and the pool sizes of intermediates of the carbon metabolism in intact leaves *in vivo* (Keerberg and Viil, 1988).

Results and discussion

Table 1 summarises the calculated rates of carbon fluxes in the biochemical system of CO_2 assimilation in the leaves of NH and CH rye at different measurement temperatures. At 25°C the rate of true photosynthesis in an atmosphere with a normal concentration of CO_2 was approximately equal in NH and CH rye. Lowering the temperature to 5°C did not change the rate of photosynthesis in CH rye, while in NH rye a decrease of about 30% was detected.

The rate of carbon flux through the glycolate cycle in CH rye was 80% higher at 25°C and about 4 times higher at 5°C than in NH rye (Table 1). The flux of carbon through the glycolate cycle is determined by the rate of oxygenation of ribulose bisphosphate (RuBP). The relative rate of oxygenation is dependent on the ratio of the CO_2/O_2 concentrations in the carboxylation centres: the lower the internal concentration of CO_2 the higher the rate of oxygenation. In CH leaves the resistance for CO_2 diffusion was always higher than in NH leaves, resulting in a decrease in the internal concentration of CO_2 (at 25°C, 9.1 and 6.0 μM in NH and CH leaves, respectively). The higher stomatal resistance in CH leaves may also be concluded from the extent of refixation of respiratory CO_2 inside the leaf: 30–40% and 70–80% in NH and CH leaves, respectively (Table 1). Higher stomatal resistance and lower internal CO_2 concentration may be regarded as the only reasons for the higher rate of carbon flux through the glycolate cycle in CH leaves. No changes were found in the CO_2/O_2 specificity of Rubisco measured at 25°C (71 and 74 in NH and CH rye, respectively).

Table 1

Rates of carbon fluxes in the biochemical system of CO_2 assimilation in leaves of non-hardened and cold-hardened winter rye at different ambient air temperatures (μg-atom C. m^{-2}. s^{-1})

Carbon fluxes	NH rye		CH rye	
	25°C	5°C	25°C	5°C
True photosynthesis	7.10±0.22	4.99±0.30	6.29±0.14	6.60±0.57
Carbon flux through the glycolate cycle	2.85±0.19	1.04±0.07	4.73±0.38	4.14±0.69
Decarboxylation of glycine in the glycolate cycle	0.71±0.05	0.26±0.02	1.18±0.10	1.04±0.17
Synthesis of				
sucrose	4.69±0.10	3.98±0.01	4.13±0.09	5.00±0.16
starch	0.40±0.02	0.18±0.01	0.09±0.01	0.06±0.01
malate	0.35±0.01	0.15±0.01	0.37±0.01	0.22±0.01
aspartate	0.19±0.02	0.08±0.01	0.26±0.04	0.11±0.01
alanine	0.11±0.02	0.05±0.01	0.11±0.01	0.12±0.01
glutamate	0.06±0.01	0.01±0.01	0.02±0.01	0.03±0.01
C_3- and C_4-acids total	0.71±0.06	0.29±0.02	0.76±0.07	0.48±0.01
Refixation of respiratory CO_2 inside the leaf (%)	35.7	29.3	67.5	76.7

Lowering the temperature from 25°C to 5°C resulted in an almost 3-times decrease in the rate of carbon flux through the glycolate cycle in NH leaves, while in CH leaves only a slight decrease in this flux was detected (Table 1). Retardation of the glycolate cycle may be the result of the suppression of the oxygenase function of Rubisco and an increase in its CO_2/O_2 specificity in response to the decrease in temperature (Brooks and Farquhar, 1985; Pärnik et al., 1995). However, the reason for the different extents of this suppression in NH and CH plants remains unclear. The possibility that the kinetic properties of Rubisco are modified during cold acclimation in a manner making its CO_2/O_2 specificity less sensitive to changes in leaf temperature cannot be excluded.

Acclimation to low growth temperatures resulted in a severe suppression of starch synthesis at both 25°C and 5°C. It was accompanied by a higher rate of carbon incorporation into malate and other intermediates of glycolysis and the tricarboxylic acid cycle (Table 1). The relative rates of sucrose synthesis (as a percentage of the rate of true photosynthesis) were equal in NH and CH rye at both temperatures. This fact suggests that the higher content of sucrose found in the photosynthesizing cells of cold-hardened leaves is not the result of more rapid synthesis but rather of the lower rate of consumption in growth processes.

The calculated values of pool sizes for photosynthetic metabolites are presented in Table 2. At 25°C the total pool of sugar phosphates was approximately equal in CH and NH leaves. A temperature shift from 25°C to

Table 2

Pool sizes and metabolite contents in the biochemical system of CO_2 assimilation in leaves of non-hardened and cold-hardened winter rye at different ambient air temperatures

Compound	NH rye		CH rye	
	25°C	5°C	25°C	5°C
Total pool of sugar phosphates (*μg-atom C. m*$^{-2}$)	431± 7	648±10	514±24	1382±203
Pool sizes (*μmol. m*$^{-2}$) of				
PGA	20± 3	28± 1	24± 4	73± 1
RuBP	12± 2	11± 1	26± 1	59± 1
HMP	38± 1	53± 1	25± 2	101± 2
Ratio of pools RuBP/HMP	0.32	0.21	1.04	0.68
Pool sizes (*μmol. m*$^{-2}$) of				
glycine	>146	72± 5	84± 2	258±88
serine	78±17	67± 9	120±28	>98
Contents (*μmol. m*$^{-2}$) of				
glycine	602±48	123±10	262±25	194±10
serine	322±10	333±17	1284±99	1347±62
Ratio of serine/glycine contents	0.53	2.71	4.90	6.94
Rate constants of glycine decarboxylation (*10^3.s*$^{-1}$)	17.2	7.3	32.8	>10.7

Abbreviations: PGA – 3-phosphoglyceric acid; RuBP – ribulose-1,5-bisphosphate; HMP – hexose monophosphates

5°C resulted in an increase in the total pool of sugar phosphates in both NH and CH rye, but to a higher extent in CH rye. This may reflect a decrease in the rate constants of the reactions converting sugar phosphates to the end products of photosynthesis. The more pronounced increase in sugar phosphates in CH leaves may be the result of the higher rate of CO_2 fixation in these leaves. The pools of phosphoglyceric acid (PGA) followed the same pattern of temperature dependence as the total pools of sugar phosphates.

At both temperatures the pools of RuBP were significantly larger in CH leaves than in NH leaves. The same was valid for the ratio of the pool of RuBP to the pools of hexose monophosphates (Table 2). These results may be interpreted as an increase in the enzymatic potential of the regenerative phase of the reductive pentose phosphate cycle. At normal CO_2 concentration this higher potential could not be manifested as a higher rate of photosynthesis due to the stomatal limitation to CO_2 fixation. In CH leaves the larger pool of RuBP compensates for the lower level of internal CO_2 to give a rate of photosynthesis equal to that in NH leaves (see Table 1, 25°C). However, under conditions where the stomata do not limit photosynthesis, for instance at high CO_2 concentrations, one can expect to find elevated rates of photosynthesis in CH leaves compared to NH ones. Indeed, in the study of Öquist et al. (1993), carried out with winter rye of the same cultivar, it was demonstrated that the CO_2- and light-saturated rate of photosynthetic O_2 evolution was about 40% higher in CH leaves than in NH leaves.

In CH leaves the active pools of serine measured at both 25°C and 5°C were larger than in NH leaves (Table 2). The same was valid for the total content of serine measured with an amino acid analyser and for the ratio of the serine/glycine contents. The apparent rate constants of glycine decarboxylation (calculated as the ratio of the rate of glycine conversion to its pool size) were also higher in CH plants (Table 2). These facts suggest that cold acclimation of winter rye leads to an increase in the activity (or content) of the glycine decarboxylase system. This adaptive feature may have a preventive significance in preventing glycine decarboxylase from becoming a limiting enzyme at elevated rates of carbon flux through the glycolate cycle in CH plants.

The part processes of the photosynthetic carbon metabolism affected during the cold acclimation of winter rye are schematically depicted in Fig. 1.

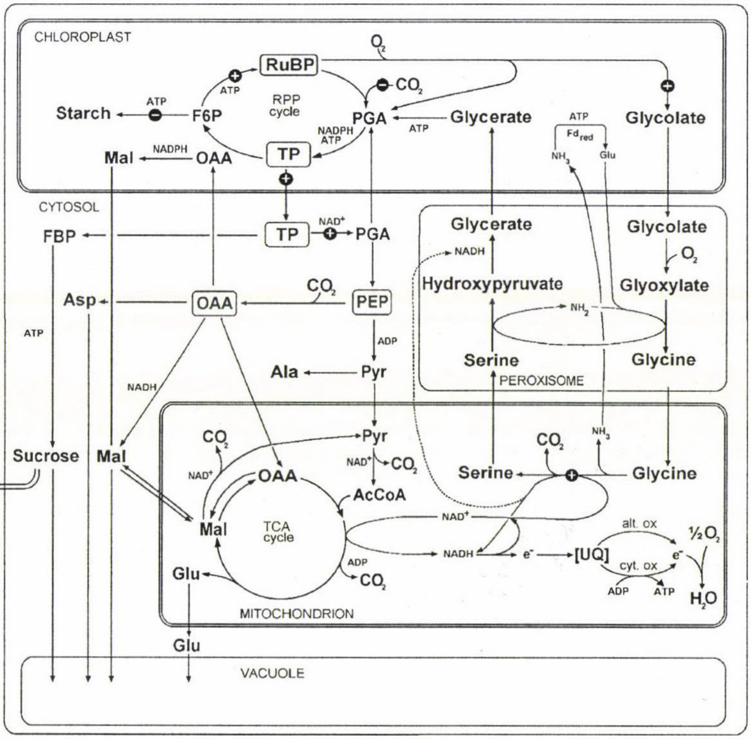

Fig. 1. Scheme of the photosynthetic carbon metabolism in C_3-plants. Reactions modified during cold acclimation are marked with the signs "+" and "–". (Abbreviations: RPP cycle, reductive pentose phosphate cycle; RuBP, ribulose-1,5-bisphosphate; F6P, fructose-6-phosphate; PGA, 3-phosphoglyceric acid; TP, triose phosphates; Mal, malate; OAA, oxaloacetate; FBP, fructose-1,6-bisphosphate; Asp, aspartate; PEP, phosphoenolpyruvate; Ala, alanine; Pyr, pyruvate, AcCoA, acetyl coenzyme A; TCA cycle, tricarboxylic acid cycle; Glu, glutamate; UQ, ubiquinone; alt. ox., alternative oxidase; cyt. ox., cytochrome oxidase)

Acclimation results in an increase in the enzymatic capacity of the regenerative phase of the reductive pentose phosphate cycle and in a corresponding increase in the rate of photosynthesis under conditions where the stomata do not limit CO_2 fixation. The higher stomatal resistance of CO_2 diffusion in CH plants leads to elevated rates of carbon flux through the glycolate cycle and of glycine decarboxylation. This is accompanied by a specific increase in the activity of the glycine decarboxylase system. In cold-hardened plants the synthesis of starch is almost entirely suppressed. Triose phosphates which could not be used for starch synthesis in CH plants are exported out of the chloroplasts to the cytosol where they are preferentially directed to the synthesis of the intermediates of respiratory pathways.

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EFFECT OF TUBULIN PROTEIN MODIFIERS ON THE WATER EXCHANGE OF NON-HARDENED AND COLD-HARDENED PLANTS OF WINTER WHEAT

L. KHOKHLOVA, O. OLINEVICH, E. PAHLICH*, N. TARAKANOVA, E. ASAFOVA and I. VOLOVNIC

DEPARTMENT OF PLANT PHYSIOLOGY, UNIVERSITY OF KAZAN, 420008, KAZAN, RUSSIA
*INSTITUTE OF PLANT PHYSIOLOGY, JUSTUS LIEBIG-UNIVERSITY, GIESSEN, D-35392, GERMANY

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It was shown that the cold hardening of plants decreased the sensitivity of the physiological and biophysical indexes of water status and the permeability of cellular membranes to structural modifiers of microtubules. These changes were most typical of the frost-resistant line of winter wheat. It was concluded that the action of low temperature on water status and the physiological state of the plants was mediated through the genetically determined ability of the cytoskeleton to became stabilized.

Key words: winter wheat, *Triticum aestivum* L., water-holding capacity, cytoskeleton, frost resistance

Introduction

Plant cell responses to low temperature depend on water status. The water-holding capacity (WHC) of tissues/cells limits the water loss of plants under freezing stress (Krasavtsev, 1988). The processes of cytoskeletal filament association and dissociation affect the structure and properties of cell water (Clegg, 1984). Microtubules and microfilaments take part in the polar transport of water in plant (Wayne and Tazawa, 1988) and animal (Snigirevskaya, 1990) cells. WHC is an integral physiological index of plant water exchange which depends on many mechanisms involving the participation of various cell structures. However, the role of the cytoskeleton in this process is unknown. The aim of the present work was to establish the dependence of WHC in tissues/cells on the structural state of tubulin microtubules under plant hardening at low temperatures.

Materials and methods

The plants of three winter wheat (*Triticum aestivum* L.) lines which differed with respect to frost resistance were the objects of investigation: Bezostaya 1 – weakly frost-resistant, Mironovskaya 808 – moderately frost-resistant, Albidum 114 – frost-resistant. The plants were grown in hydroculture, illumination – 100 W/m^2, photoperiod – 12 h. Non-hardened plants were grown at 23°C for 5 or 9 days; hardened plants were kept at 3°C for 3 or 7 days at the age of 4 or 8 days, respectively. The structural modifiers of microtubules – colchicine (1 mM), oryzalin (15 mkM) and dimethyl sulphoxide (DMSO, 7%) – were infiltrated in intact tissues. WHC was determined as the quantity of water remaining in the tissues after hypertonic treatment with a 20% solution of PEG-6000. Using the impulse method of NMR measurements were made on the biophysical indexes of water intercellular transport - times of spin-spin relaxation (T_2) and the effective coefficient of water self-diffusion (D_{eff}). The permeability of the membranes and the physiological state of the plants were tested by electrolyte leakage from the tissues.

Results and discussion

The treatment of tissues with colchicine caused a greater reduction in WHC in cold non-hardened plants of weakly and moderately frost-resistant wheat lines (Bezostaya 1 and Mironovskaya 808) than in the frost-resistant line (Albidum 114). DMSO, by contrast, induced the greatest increase in WHC in Albidum 114 (Fig. 1). The joint treatment of tissues with DMSO and colchicine led to the disappearance of the inhibitory effect of colchicine on WHC, as a consequence of which the leakage of water from tissues in a 20% solution of PEG-6000 decreased and WHC increased in these variants (colchicine+DMSO) as compared with the action of colchicine alone. Colchicine is an antimitotic compound which blocks the polymerization of tubulin proteins, prevents the lengthening of MTs and induces their decomposition (Morejohn, 1991). DMSO stabilizes cortical MTs in higher plants (Hahne and Hoffman, 1984). At high concentrations it is capable of inducing tubulin polymerization and acts as a cryoprotector (Himes et al., 1977). The colchicine-induced disorganization of the cytoskeleton is accompanied by the dehydration of cytoskeleton filaments and other cellular structures bound to them. The decrease in bound water content and the increase in free water (the rise in water potential) may be a consequence of these changes. The latter is obviously the main reason for the decrease in the WHC of the tissues due to the colchicine effect. On the other hand, DMSO stabilizes the cytoskeleton and enhances water binding (the decrease in water potential) and this is reflected in an increase in WHC. DMSO effectively neutralizes the negative influence of colchicine on the WHC of the tissues.

Changes in WHC in non-hardened plants correlate well with the results of experiments on electrolyte leakage (Fig. 1). Colchicine caused an increase in cellular membrane permeability, while DMSO decreased it. Consequently colchicine promoted damage to the plants, whereas DMSO stabilized their physiological state. After the joint treatment of tissues with DMSO and colchicine the permeability of the membranes remained at a fairly high level. However, DMSO somewhat decreased the colchicine-induced electrolyte leakage, especially in Bezostaya 1 and Mironovskaya 808, while it had little effect on the colchicine-induced membrane permeability in Albidum 114 (compare variants colchicine+DMSO and colchicine).

The sensitivity of WHC and electrolyte leakage to colchicine and DMSO in cold-hardened plants decreased as compared with non-hardened plants (Fig. 1). These results can be attributed to the fact that in cold-hardened cells tolerant populations of MTs appear with less affinity to influencing agents, or to the synthesis of new protective proteins which stabilize the cytoskeleton. This supposition is founded on reports (Hogetsu, 1986; Kerr and Carter, 1990) about the localization in one and the same cell of MTs with different cold resistance and about selective changes in these under cold stress. DMSO produced a greater increase in WHC in cold-hardened plants of Albidum 114 than in Bezostaya 1 or Mironovskaya 808. DMSO suppressed colchicine-induced changes in WHC, including damage to the membranes. But these effects of DMSO were weaker in cold-hardened plants than in non-hardened ones. The common property for both types of plants was that DMSO was more effective as a stabilizer of cellular membranes in less frost-resistant lines of wheat.

Among the antimitotic compounds, oryzalin (a dinitroaniline herbicide) has high specificity to the MTs of plant cells. Oryzalin binds with tubulin, inhibits the polymerization of MTs and induces their disappearance in cells of higher plants (Morejohn, 1991). It was shown that the treatment of tissues with oryzalin for 3 hours led to the same decrease in WHC (Table 1) as a 24-hour treatment with colchicine (Fig.. 1). The WHC of non-hardened and cold-hardened plants of a frost-resistant wheat line gave a poorer response to oryzalin than the WHC of the other two lines. There is thus a similarity between the actions of oryzalin and colchicine.

Oryzalin changed the relaxation and diffusive parameters of water in the tissues (Table 1). Oryzalin caused a decrease in the spin-spin relaxation times (T_2) which was smaller in Albidum 114. The rate of water membrane transfer contributes greatly to the value of T_2 (Gusta et al., 1979; Anisimov et al., 1982). The decrease or increase in T_2 may be the consequence of an increase or decrease in the water exchange rate across the plasma membrane.

Table 1

Effect of oryzalin on physiological (WHC) and biophysical (T_2, D_{eff}) indexes of water exchange and on electrolyte leakage (EL) in winter wheat plants

Variants	Non-hardened (23°C)				Cold-hardened (3°C, 7 days)		
	WHC	T_2	$D_{eff}\times 10^{-5}$	EL	WHC	T_2	$D_{eff}\times 10^{-5}$
			Bezostaya 1				
Control	63.8±0.7	77.3±2.7	0.38±0.01	10.7	66.7±0.6	–	–
Oryzalin	56.3±1.0	70.4±1.2	0.58±0.03	12.5	61.3±0.9	–	–
			Mironovskaya 808				
Control	67.8±0.8	77.4±1.3	0.45±0.06	7.2	70.0±0.7	84.2±1.2	0.26±0.04
Oryzalin	62.3±0.9	71.8±1.5	0.50±0.04	10.2	63.8±0.7	79.9±0.9	0.38±0.05
			Albidum 114				
Control	64.8±0.8	71.0±1.5	0.51±0.04	4.0	74.1±0.8	78.7±1.0	0.32±0.04
Oryzalin	62.1±1.3	67.2±1.3	0.51±0.06	3.2	74.1±1.0	78.6±1.2	0.32±0.05

Tissues exposed to oryzalin (15 mkM) solution for 3 h, WHC as % of fresh weight, T_2 in min., D_{eff} in $cm^2\ s^{-1}$, EL as % of total leakage

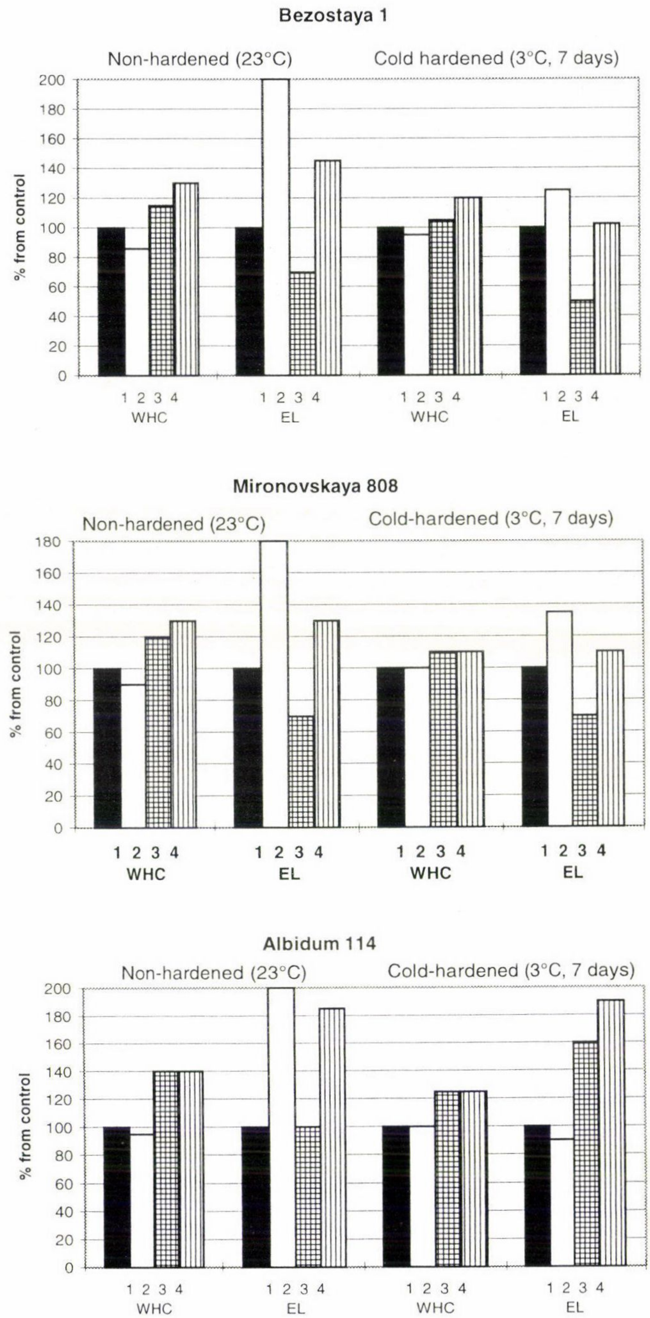

Fig. 1. Effect of colchicine and DMSO on water-holding capacity (WHC) and electrolyte leakage (EL) : 1. control, 2. colchicine (1mM), 3. DMSO (7%), 4. colchicine (1 mM) + DMSO (7%). Tissues exposed to solutions of colchicine and DMSO for 24 h.

Taking this fact into consideration it can be said that oryzalin caused the greatest acceleration in water transport in the cells of less frost-resistant lines. The cold-hardening of the plants weakened this effect of oryzalin in Mironovskaya 808 plants or completely overcame it in Albidum 114. The cause of the acceleration of oryzalin-induced water transport may be connected with the increase in membrane permeability as the result of the breakdown of interactions between cortical MTs and the water channels of the plasma membrane.

The effective self-diffusion coefficient (D_{eff}) helps to evaluate the diffusive permeability of cells and tissues for water (Anisimov, 1982). The effect of oryzalin led to an increase in D_{eff} in Bezostaya 1 and Mironovskaya 808 and did not change the D_{eff} of Albidum 114. In cold-hardened plants D_{eff} was lower and T_2 higher than in non-hardened plants. This shows that the cold hardening of plants slows down the water transport across the plasma membrane. Oryzalin increased the electrolyte leakage from tissues of less frost-resistant lines and somewhat decreased the electrolyte leakage from tissues of the frost-resistant line (Table 1). Consequently, oryzalin exerted more intense damage on plants of Bezostaya 1 and Mironovskaya 808 than on Albidum 114. This correlates with the results on changes in WHC, T_2 and D_{eff}.

Summing up the results achieved in this work, it can be concluded that under conditions inducing the depolymerization of MTs (colchicine, oryzalin) WHC decreases and this is correlated with an increase in cellular membrane permeability for water and ions. On the other hand, under conditions favourable for the polymerization of MTs (DMSO, hardening) WHC increases, the rate of water transport decreases and the barrier properties of the plasma membrane are enhanced. This suggests that the thermodynamic state of water and its intercellular transport are controlled by the integrity of the cytoskeletal network. The disorganization of the tubulin cytoskeleton decreases water binding, causes dehydration and increases water transport across the plasma membrane. If this process continues it may lead to critical water loss in the cells.

It was shown that the cold hardening of plants decreased the sensitivity of the physiological and biophysical indexes of water status (WHC, T_2, D_{eff}) and the permeability of cellular membranes to structural modifiers of tubulin MTs. This raised the possibility of the appearance of new cold-tolerant populations of MTs in cold-hardened cells. The greater loss of sensitivity of the measured parameters is typical of the frost-resistant line of wheat. The lowered sensitivity of WHC to the inhibitors of tubulin protein polymerization is suggested as a marker of MT cold stability.

The hypothesis that the action of low temperature on water status and the physiological state of plants is mediated through the genetically determined ability of the cytoskeleton to become cold-stabilized requires further investigation. The conclusion about the higher efficiency of DMSO as a stabilizer of membranes in less frost-resistant lines of winter wheat could be useful in practice.

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EFFECT OF LIGHT ON *IN VITRO* TUBERIZATION OF POTATO OF PURE *SOLANUM TUBEROSUM* ORIGIN

J. DOBRÁNSZKI

AGRICULTURAL RESEARCH INSTITUTE OF DEBRECEN AGRICULTURAL UNIVERSITY, NYÍREGYHÁZA, WESTSIK V. U. 4–6, H–4400, HUNGARY

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The effects of an environmental factor, light, on *in vitro* tuberization were analysed on three potato cultivars of similar genetic origin and in the same maturity group. No growth regulators were added to the culture medium to avoid the potential effect(s) of growth regulators on the response to environmental stimuli. Tuberization was induced by short-day treatment after culturing the plantlets for 4 weeks under long days. The effect of different combinations of short days and dark treatment and of short days with different light intensity, together with the relationship between them, were examined with respect to the tuberization response.

The dark treatment affected the rate of tuber initiation. Its influence depended on cultivars and light intensity. It also had a synchronizing effect on tuber initiation, especially at high light intensity. The effect of light applied during short days largely depended on the duration of the dark treatment. Light applied after induction delayed tuberization. The rational *in vitro* application of light as an environmental factor controlling the tuberization process of potato *in vivo* has made it possible to reach a tuberization rate at least as high or higher than with any method described previously based on the use of growth regulators to induce tuberization.

Key words: photoperiod, light intensity, *Solanum tuberosum* origin, tuber development and number

Introduction

The tuberization of potato is a complex developmental process affected by both environmental and endogenous factors, both of which are well-regulable under *in vitro* conditions.

Most of the work published on the *in vitro* tuberization of potato has focused on using growth regulators for tuber induction. Many regulating substances have been investigated (Palmer and Smith, 1969; Garcia-Torres and Gomez-Campo, 1973; Wang and Hu, 1982; Mangat et al., 1984; Estrada et al., 1986; Jelaska et al., 1987; Ortiz-Montiel and Lozoya-Saldana, 1987; Rosell et al., 1987; Lentini et al., 1988; Lentini and Earle, 1991). However, the application of these growth regulators did not allow the innate tuberization capacity of the potato plants to be expressed, resulting in a relatively low tuber number per plant (generally less than 1.0) (Garcia-Torres and Gomez-Campo, 1973; Wang and Hu, 1982; Tovar et al., 1985).

Several authors have studied the effect of environmental factors such as light (Slimmon et al., 1989; Lentini and Earle, 1991; Pelacho and Mingo-Castel, 1991; Perl et al., 1991; Nowak and Asiedu, 1992; Seabrook et al., 1993; Pelacho et al., 1994) and temperature (Bohac et al., 1988, Nowak and Colborne, 1989;

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Harvey et al., 1992) on *in vitro* tuberization. However, they examined the effects of these factors in addition to the use of growth regulators in the medium. This means that the environmental factors had permitting effects rather than regulating ones on tuberization. The growth regulators applied in the medium had the regulating role.

Recently there have been some reports concerning the effects of light or temperature on *in vitro* tuberization without using growth regulators (Hussey and Stacy, 1984; Thieme, 1988/89; Garner and Blake, 1989; Charles et al., 1992; Charles et al., 1993; Dobránszki and Mándi, 1993; Akita and Takayama, 1994; Dobránszki, 1996). In these systems the potential effect(s) of regulators on the response to environmental stimuli can be avoided.

The present study was undertaken to examine the tuberization characteristic of *in vitro* plantlets of pure *Solanum tuberosum* origin at different light intensities applied in combination with various photoperiodic treatments. The aim of the experiments was to determine the basic physiological processes which could then be applied to develop a mass-tuberization system in order to improve the tuberization percentage.

Materials and methods

Plant material

In vitro cultures of *Solanum tuberosum* L. cv. Gülbaba, Nyírségi rózsa and Somogy gyöngye (mid-early) were used. These have pure *Solanum tuberosum* origin and Gülbaba was one of the parents of Nyírségi rózsa. The plant material was maintained as an *in vitro* culture.

Shoot culture (phase 1)

Nodal cuttings of four-week-old *in vitro* plants grown in Kilner jars served as explants in the experiments. The initial explants were placed in Kilner jars (400 ml, 75 mm × 85 mm) covered with plastic caps. The experiments were carried out in jars containing 30 nodal explants on 40 ml of medium containing the salt mixture of Murashige–Skoog (1962) supplemented with 0.8% agar-agar and 3% sucrose.

The cultures were grown for four weeks in a culture room at 24/15°C day/night temperature, 16 h photoperiod and 8,000 lux light intensity. On average 27 fully developed plantlets (at least 4 cm long with a large leaf surface and well-developed roots) per jar could be grown after long-day treatment for four weeks.

Tuber induction treatment (phase 2)

Tuber induction treatments were carried out after four weeks of long-day treatment, because this period is required to reach vigorous plant development *in vitro* (Garner and Blake, 1989; Charles et al., 1992), which is an essential precondition for the perception of environmental stimuli such as light (Smith, 1982; Hammes and Beyers, 1973).

Tuberization was induced on medium with a layer of 8% sucrose solution poured onto the cultures. Five different combinations of illumination (photoperiodic treatments Fp 1–5) were used, involving different combinations of short days (8 h illumination/day) and total darkness (0 h illumination/day) (Table 1).

Three different light intensities were applied during the short-day treatments: 8000 lux (Li–0 treatment), 4000 lux (Li–1 treatment), 400 lux (Li–2 treatment).

Table 1
Illumination combinations applied after a four-week culture period under long days

Treatment	Weeks		
	Short days (8 h)	Darkness (0 h)	Short days (8 h)
Fp–1	13	–	–
Fp–2	2	2	9
Fp–3	2	11	–
Fp–4	1	12	–
Fp–5	0+3 days	11+4 days	–

Measurement and data analysis

The time of tuber initiation *in vitro* was determined by recording the appearance of the first microtuber in each jar. The tuber development was recorded by counting the number of tubers per jar every 2 weeks after tuber initiation and by observing the morphological type of the tubers developed. The tuber number per jar and the size of microtubers in different treatments was evaluated at the end of the experiment (after 17 weeks).

The data of tuber initiation time and tuber number were statistically analysed by variance analysis. At least 10 replicates of each treatment per clone were evaluated for the effect of the treatments. The analyses were conducted using SPSS PC+4 software.

Results and discussion

Tuber initiation

The time when tubers were initiated varied with the cultivar and with different combinations of illumination and light intensity (Figs. 1–3).

Exposure to darkness after short days caused an acceleration in tuberization. The Fp–2 treatment caused significantly earlier tuber initiation in the case of Somogy gyöngye at high light intensity (Li–0 treatment: 8000 lux) compared to the Fp–1 treatment. Nyírségi rózsa also produced tubers earlier in the Fp–3 treatment compared to the Fp–1 treatment. Treatments Fp–4 and Fp–5 did not cause a further decrease in the number of days required to initiation in the case of these cultivars. Only the Fp–5 treatment caused significantly earlier tuber initiation in the case of Gülbaba (Fig. 1).

The dark treatment applied in different illumination combinations with short days had another important effect on tuber initiation. It had a synchronizing effect that depended on the clones and the Fp treatments. The Fp–2 and Fp–3 treatments caused a significant decrease in the standard deviation(s) of the data in the case of Gülbaba and Somogy gyöngye. In the case of Nyírségi rózsa only the Fp–4 treatment caused a significant decrease in the standard deviation of the data and the extension of darkness by reducing the period of short-day treatment applied before darkness (Fp–5 treatment) caused a cessation of its synchronizing effect (Fig. 1).

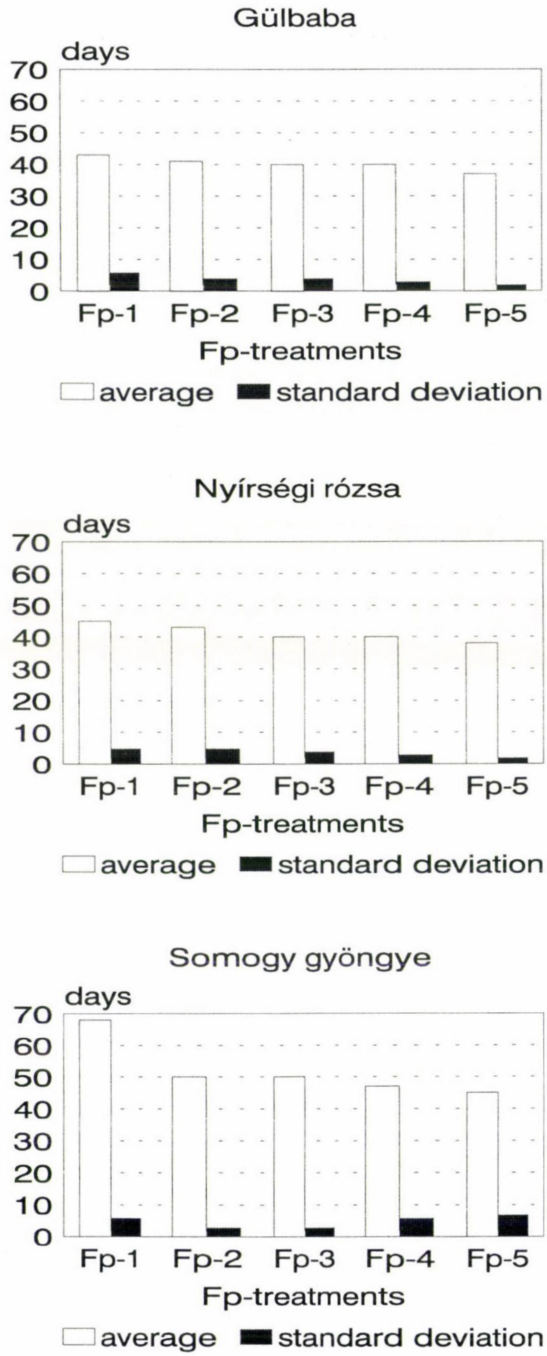

Fig. 1. Number of days required for the appearance of the first tuber in each jar at high light intensity (8000 lux) applied during short days

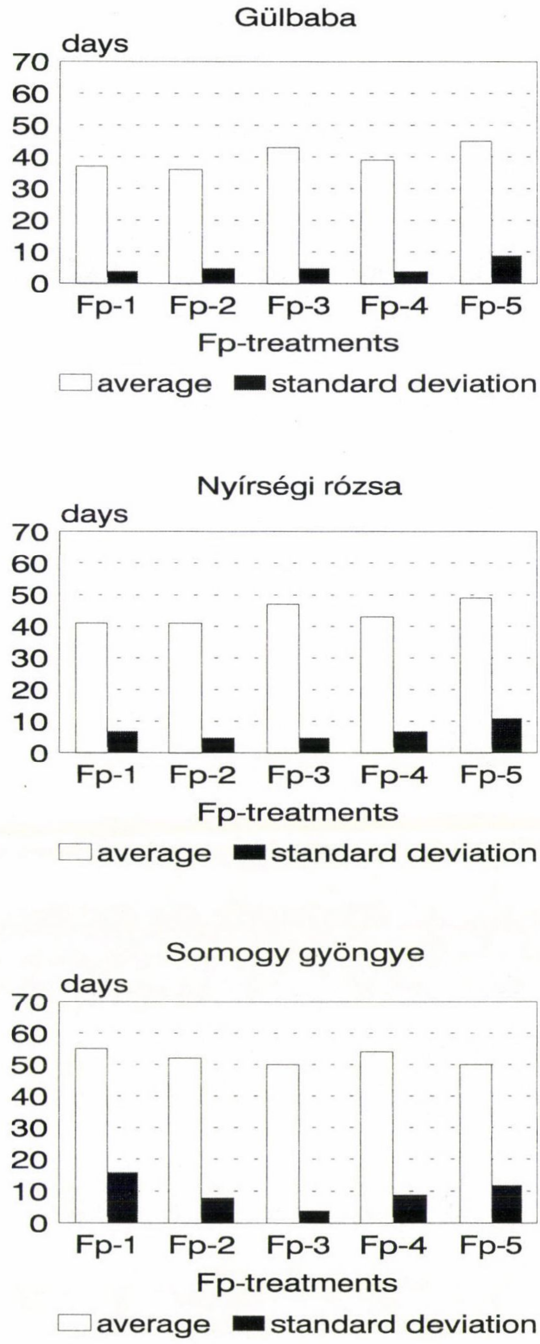

Fig. 2. Number of days required for the appearance of the first tuber in each jar at medium light intensity (4000 lux) applied during short days

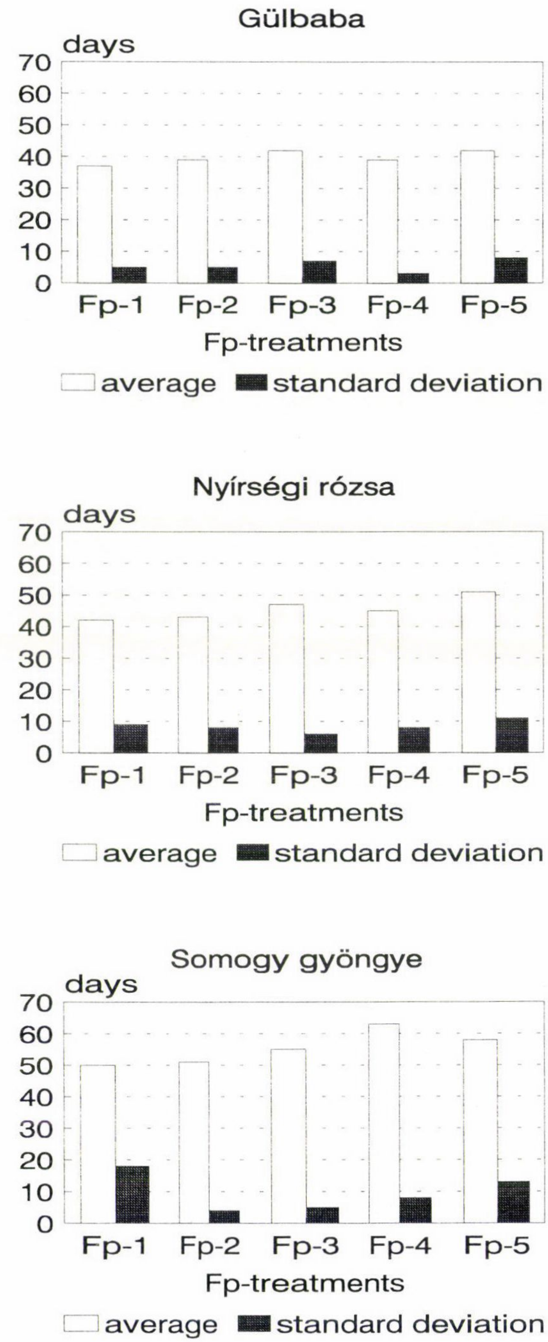

Fig. 3. Number of days required for the appearance of the first tuber in each jar at low light intensity (400 lux) applied during short days

At medium light intensity (Li–1 treatment: 4000 lux) the effect of darkness applied in the Fp–2–5 treatments depended on the genotype (Fig. 2). It had a delaying effect on tuber initiation in the case of Gülbaba in treatments Fp–3 and Fp–5 and in the case of Nyírségi rózsa in the Fp–3 treatment. It had no significant effect in the case of Somogy gyöngye. The synchronizing effect of darkness was also noticeable at this light intensity in the case of Somogy gyöngye in treatments Fp–2 and Fp–3, although to a lesser degree than at high light intensity. However, the further extension of darkness caused the cessation of its synchronizing effect and even in the case of Gülbaba and Nyírségi rózsa it resulted in an increase in the standard deviation of the data in the Fp–5 treatment.

At low light intensity (Li–2 treatment: 400 lux) the dark treatment had neither a synchronizing, nor an accelerating effect on tuber initiation (Fig. 3).

In the Fp–1 treatment high light intensity caused significantly later tuber initiation in the case of Somogy gyöngye and Gülbaba in the case of lower light intensities (treatments Li–1–2). The synchronization of tuber initiation was detectable in the case of Somogy gyöngye at high light intensity.

In the Fp–2 treatment there was no significant difference between the light intensity treatments in the time of tuber initiation in the case of Somogy gyöngye and Nyírségi rózsa, but in Gülbaba initiation occurred significantly later at high light intensity (Li–0 treatment). The standard deviation of the data was considerably smaller at high light intensity (Li–0 treatment) in the case of Somogy gyöngye

In the Fp–3 treatment there was no significant difference between the light intensity treatments in the time of tuber initiation in the case of Gülbaba, but the initiation of Nyírségi rózsa occurred significantly later at lower light intensity (treatments Li–1–2) and that of Somogy gyöngye at the lowest light intensity (Li–2 treatment).

There was no great difference between the standard deviations of the data in the case of two clones (Somogy gyöngye, Nyírségi rózsa), but it was smallest at high light intensity (Li–0 treatment) in the case of Gülbaba.

In treatment Fp–4 there was no significant difference between the times required for the appearance of the first tuber in each jar in the case of Gülbaba, but the other two clones produced *in vitro* tubers first at high light intensity. The standard deviation of the data was not substantially different in the case of Gülbaba, but it was smaller at high light intensity in the case of the other two clones.

In the Fp–5 treatment the appearance of the first tuber occurred significantly earlier at high light intensity in the case of two clones (Gülbaba, Nyírségi rózsa), while there was no significant difference between the Li-treatments in the case of Somogy gyöngye.

In order to detect the relationship between the treatments multiple analysis of variance (MANOVA) was applied. This analysis proved that both the main effects and 2-way interactions (Fp–Li, Fp–Clones, Li–Clones) were significant ($P < 0.01$) as was the 3-way interaction (Fp–Li–Clones).

It can be concluded that light (short-day treatment) applied after the induction phase had a delaying or inhibiting effect on tuber initiation, while darkness applied after the induction stage accelerated and synchronized the tuber initiation. However, these effects of the dark treatment depended on the light intensity applied during the short days. At lower light intensities (treatments Li–1–2) the accelerating effect of darkness was not general and it even had a delaying effect on initiation in some cases. There was also a noticeable synchronizing effect of high light intensity depending on the Fp treatments.

The duration of darkness applied in different illumination treatments showed a close exponential correlation ($r^2 = 0.9634$) with the light intensity applied during short days. This means that the longer the duration of darkness is in the Fp treatment the higher the light intensity required during short days in order to reach earlier tuber initiation.

However, there was a favourable effect on tuber initiation if the cultures were illuminated by high light intensity during the induction phase before being placed in darkness (treatments Fp–3–4–5 depending on cultivars). Dark treatment applied excessively early after light treatment (treatment Fp–5) delayed initiation. This unfavourable effect of darkness was particularly pronounced at lower light intensity. The results suggest that a given quantity of light is necessary to trigger a general state of induction, but that further light effects may be inhibitory. These data and the significant 2- and 3-way interactions indicate that the light (Fp and Li) treatments always need to be optimalized for the given genotype.

Tuber development

Figures 4–6 include the tuberization curves of the cultivars examined in different Fp and Li treatments.

At high light intensity the dark treatments led to a significant increase in the number of tubers per jar, which can be seen from the second week after induction in the Fp–2 and Fp–3 treatments and from the first week and from the third day after induction in the Fp–4 and Fp–5 treatments, respectively. However, the exposure of the cultures to the dark too early (treatments Fp–4 and Fp–5) resulted in a retardation in the increase in tuber number.

At lower light intensities (treatments Li–1 and Li–2) the increase in tuber number slowed down and in the Fp–5 treatment the curves did not reach the saturation phase. Tuber initiation occurred earlier in the Fp–1 treatment and later in the Fp–5 treatment as compared to that occurring at the highest light intensity.

The morphological type of the tubers developed was determined according to McGrady et al. (1986). At given light intensity, extending the dark treatment to the end of the experiment resulted in an increase in the rate of sessile tubers in comparison with the rate of non-sessile ones, which may indicate a stronger tuberization stimulus.

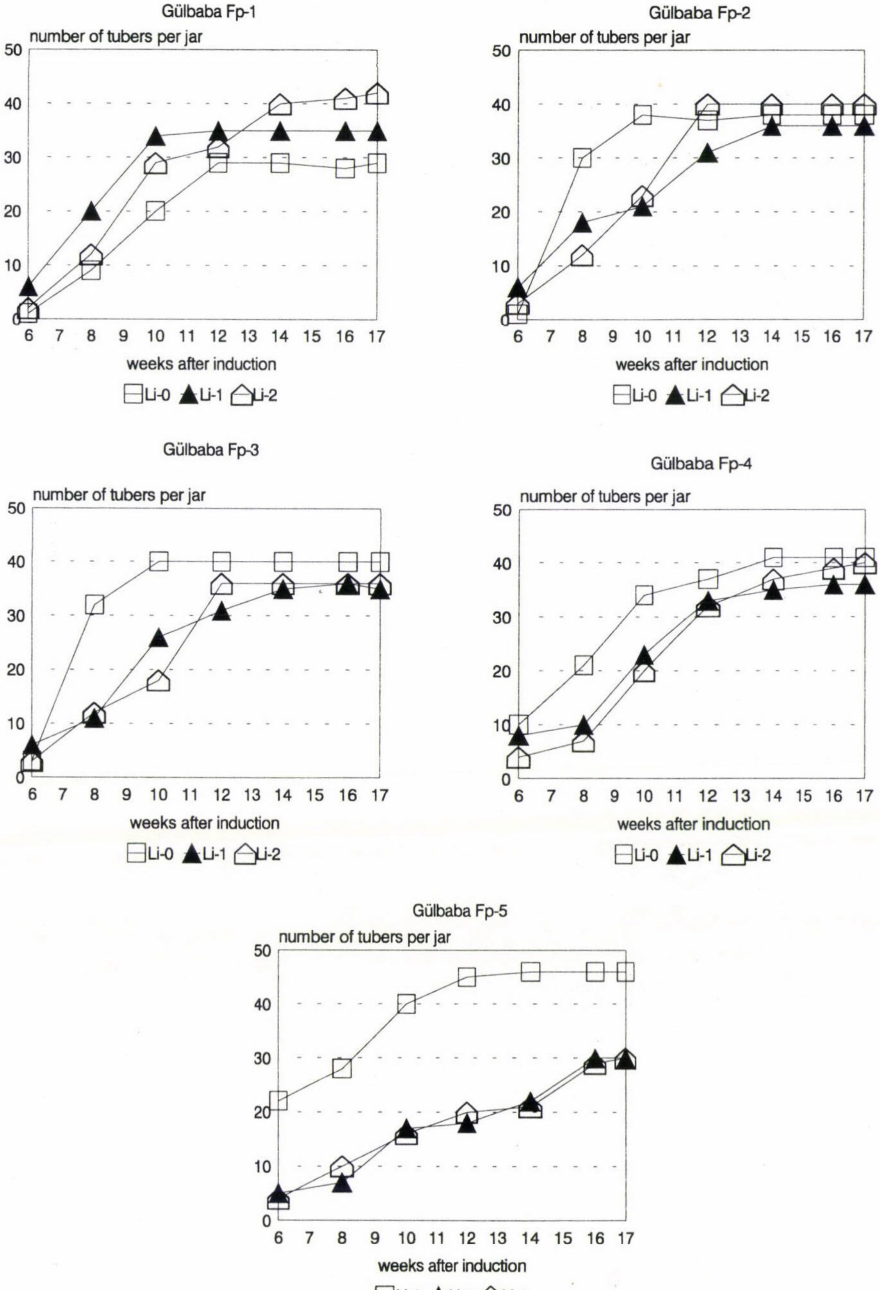

Fig. 4. Development of microtubers of cv. Gülbaba after induction

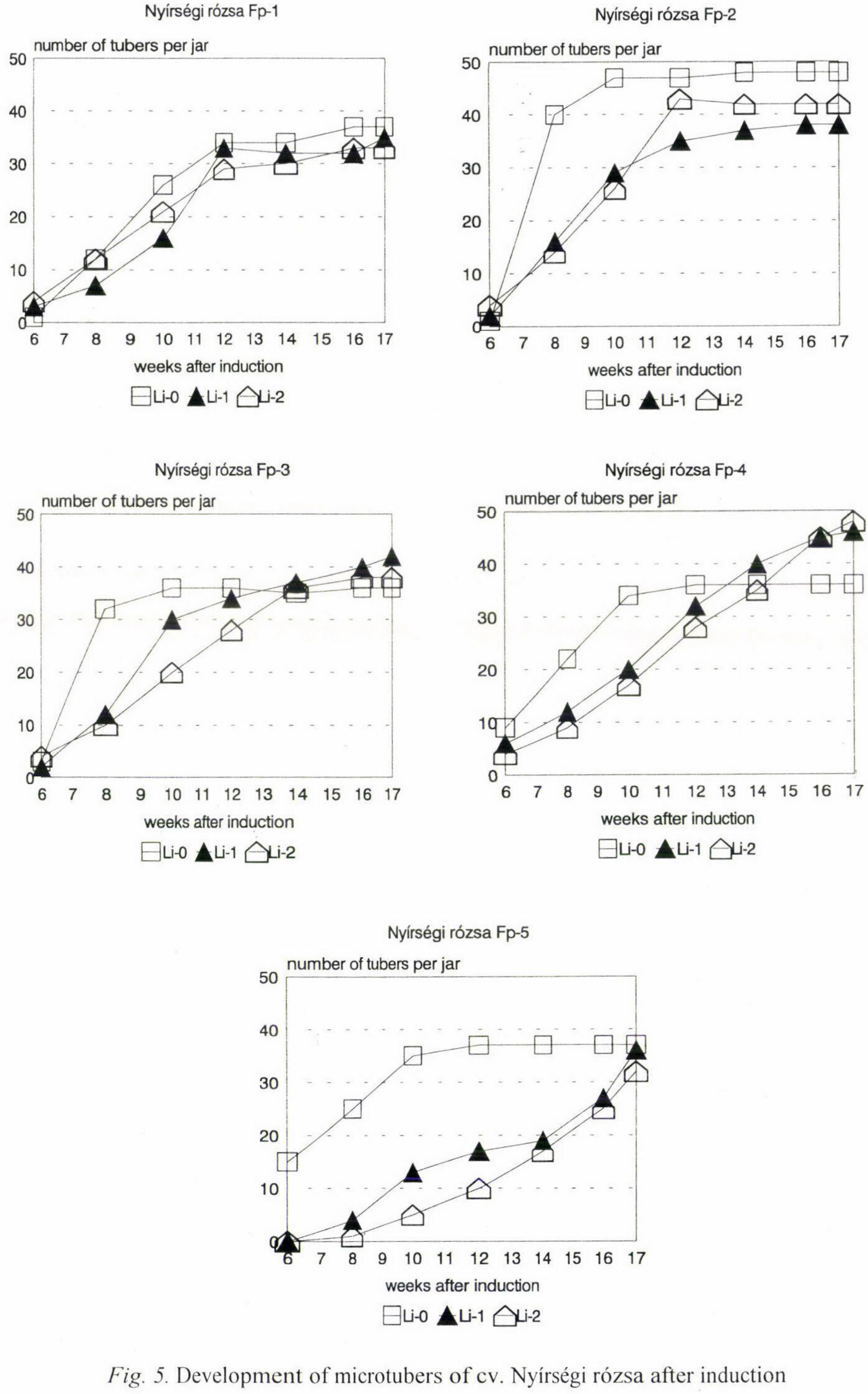

Fig. 5. Development of microtubers of cv. Nyírségi rózsa after induction

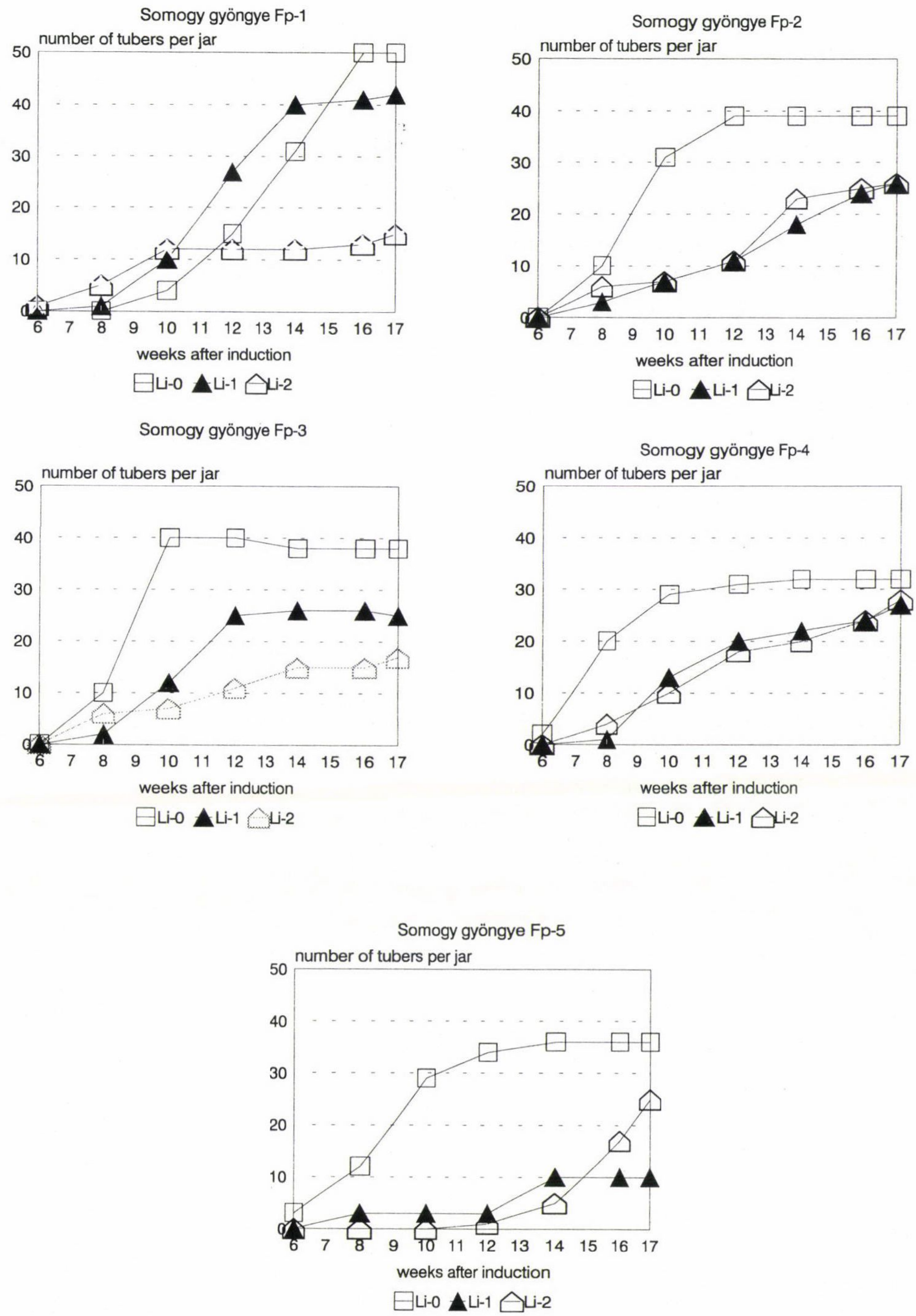

Fig. 6. Development of microtubers of cv. Somogy gyöngye after induction

The tubers produced in the Fp–1 and Fp–2 treatments were formed either directly under or partly in the medium. In other cases the plants developed long stolons and tubers developed at the end of the stolons directly under the plastic caps of the jars.

Microtubers developing within the medium had prominent lenticels, a large diameter varying from 0.7–1.8 cm and a shape similar to that of tubers formed in the field. The colour of these tubers varied from green to blackish green, probably due to the fact that they developed predominantly under light conditions. Their skin often split open, preventing their further use.

Microtubers produced in the Fp–3–5 treatments were positioned high above the surface of the medium and had an approximately round shape irrespective of the genotype. Their colour was similar to those formed in the field, varying from yellowish-white to dark rose-red. However, their diameter was smaller than for those produced under light conditions; the loss caused by water-loss of tubers was more less. These results are similar to those reported by Slimmon et al. (1989) and Nowak and Asiedu (1992).

Tuber number

Both the total number of *in vitro* tubers developed and the number of tubers 2 or >2 mm in diameter was analysed by MANOVA. Table 2 includes the average number of microtubers 2 or >2 mm in diameter per jar. The analyses proved close interactions between the treatments and between the treatments and clones. The 3-way interaction was also significant ($P < 0.01$).

Table 2
Effect of different treatments on the average number of *in vitro* tubers larger than 2 mm in diameter in each jar

Treatments	Fp–1	Fp–2	Fp–3	Fp–4	Fp–5
			Gülbaba		
Li–0	28	35	34	36	40
Li–1	35	28	33	33	28
Li–2	37	34	35	37	25
			Nyírségi rózsa		
Li–0	30	32	31	33	36
Li–1	31	36	36	35	29
Li–2	33	25	31	41	26
			Somogy gyöngye		
Li–0	37	32	32	31	31
Li–1	38	25	17	20	7
Li–2	16	20	9	25	19

At high light intensity the dark treatment (treatments Fp–2–5) resulted in significantly more tubers as compared to treatment Fp–1 in the case of Gülbaba and Nyírségi rózsa. However, the behaviour of Somogy gyöngye was quite the opposite: it produced the most microtubers in the Fp–1 treatment.

At medium light intensity the significantly lowest number of tubers were developed in the Fp–5 treatment in the case of Gülbaba and Somogy gyöngye. The tendency was similar in the case of Nyírségi rózsa, but the differences were not significant. An interruption of the dark treatment (treatments Fp–2) was also unfavourable for tuber number.

At the lowest light intensity the Fp–4 treatment was more favourable concerning the number of tubers 2 or >2 mm in diameter in the case of all the cultivars examined.

It can be concluded that in several instances more than one tuber per plant, was formed, considering only tubers larger than 2 mm in diameter. The tuber number varied from 1.41 to 1.52 depending on the cultivar in the best treatments. The best treatment means the rational *in vitro* application of light as an environmental factor controlling the tuberization process of potato *in vivo*. The best treatment was the Fp–5/Li–0 treatment combination in the case of Gülbaba, the Fp–4/Li–2 treatment combination in the case of Nyírségi rózsa and the Fp–1/Li–1 or Li–0 treatment combination in the case of Somogy gyöngye. These results are better than those attainable by using growth regulators to induce *in vitro* tubers.

The other benefit of an *in vitro* system free of growth regulators is that the genetic stability of the cultured plants can be preserved better than when growth regulators are added to the induction medium. Besides the undesirable carry-over effects on morphology, the dormancy and sprouting of the microtubers are avoidable.

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PRODUCTION OF DWARF *RUDBECKIA* USING CULTAR

M. GAD and L. GERZSON

UNIVERSITY OF HORTICULTURE AND FOOD INDUSTRY
VILLÁNYI ÚT 35–43, BUDAPEST, HUNGARY

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In order to produce a dwarf *Rudbeckia* for pot purposes, a tall variety of *Rudbeckia laciniata* L. (150–180 cm) was used. Rooted tip cuttings were treated with Paclobutrazol (Cultar) as foliar spray or as soil drench. The foliar spray treatments were 0.00, 250, 500 and 1000 ppm active ingredient (a.i.) per pot and the soil drenches 0.00, 1.0 and 2.0 mg. All the treatments used decreased plant height. The decrement was due to short internodes not to the number of nodes. Foliar spray treatments at high concentrations were more effective than soil drench treatments. The numbers of leaves and flowers were not significantly affected by the Cultar treatments. Plant fresh weight and flower pedicel length were significantly decreased by both methods. Spray treatments at 500 and 1000 ppm produced a suitable plant height for pots.

Key words: growth retardant, Paclobutrazol, Cultar, *Rudbeckia*

Introduction

Rudbeckia is an important group of hardy herbaceous perennial and annual plants which are of considerable decorative value in the garden particularly during the late summer and early autumn months.

The most popular of the tall kinds is *Rudbeckia laciniata* L. This handsome perennial plant reaches a height of 150 cm to 180 cm in the field. In order to develop a flowering pot plant from *Rudbeckia*, Paclobutrazol (Cultar) was used to produce dwarf plants.

Materials and methods

This work was carried out in the experimental farm of the University of Horticulture and Food Industry (Soroksár, Hungary).

Rooted tip cuttings (about 15 cm) of *Rudbeckia laciniata* L. were planted in 9 cm pots on 1st July 1995. The growing medium contained equal parts of peatmoss and clay. On 10th July 1995 (ten days after planting) the plants were treated with Paclobutrazol (Cultar) as foliar spray or as soil drench. The foliar spray treatments, containing 0.00, 250, 500 and 1000 ppm in aqueous solution, were applied using a low volume hand pump sprayer. The plants were sprayed until the solution ran off. The soil surface was covered with polyethylene to protect it from the solution.

The soil drench applications consisted of 0.00, 1.0 and 2.0 mg as active ingredient (a.i.), dissolved in 30 ml of water per pot. The plants were fed with 0.3% Volldünger (14:7:21+2) containing 14% ammonium, 7% P_2O_5, 21% K_2O, 1% MgO and 1% (Fe, Cu, Mn, B and Zn).

On 6th September, measurements were made on the plant height from the soil surface to the top of the tallest flower, the internode length, and the numbers of branches, leaves and nodes per plant. The fresh weights of the leaves and roots (hand washed from medium) and of the plant

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as a whole were recorded 80 days after the treatment date. Flower measurements included the number of flowers per plant, flower diameter (cm) and flower pedicel length (cm).

The experimental design was a randomized complete block with four replications. All data were subjected to analysis of variance, and means were separated using a least significant difference (LSD) test (Dawdy and Stanley, 1983).

Results

Plant height was reduced after using Cultar. As the concentration increased, the plant height decreased. The decrement percentages were 54.1, 59.6 and 66.7% after the plants were sprayed at 250, 500 and 1000 ppm, respectively (Table 1) compared with the control plants. Cultar applied as soil drenches (Table 2) reduced plant height to 71.5 cm (1.0 mg) and 63.25 cm (2.0 mg) compared to that of untreated plants (94.0 cm). The shortest plant height was obtained at 1000 ppm.

The effect of Cultar, either sprayed or applied as a soil drench, on the numbers of branches, nodes and leaves was insignificant (Tables 1 and 2).

Figure 1 illustrates the fact that *Rudbeckia* treated with Cultar had short internodes. Spraying the plants with Cultar was more effective than applying a soil drench (Fig. 2). Plants sprayed with Cultar had internode lengths of 8.83, 7.25 and 6.0 cm at 250, 500 and 1000 ppm, respectively. With the soil drench these values were 13.72 and 11.58 cm at 1.0 mg and 2.0 mg, respectively compared to 17.33 cm for the untreated plants (Fig. 3).

Leaf fresh weight was significantly increased by Cultar sprayed at 500 and 1000 ppm (Table 1) or added as a soil drench at 2.0 mg (Table 2) compared to the control plants. The weights were 2.29, 2.42 and 1.89 g at 500 ppm, 1000 ppm and 2.0 mg compared to 1.73 g for untreated plants.

Table 1

Effect of Cultar used as foliar spray on growth and flowering of *Rudbeckia laciniata*

Characters	Treatments				
	Control	250 ppm	500 ppm	1000 ppm	$LSD_{0.05}$
Plant height (cm)	94.00	46.00	38.00	31.30	8.52
No. of branches	5.50	5.25	5.00	5.25	NS
No. of nodes	5.50	5.25	5.25	5.25	NS
No. of leaves	3.75	4.00	4.25	4.00	NS
Leaf fresh weight (g)	1.73	2.11	2.29	2.42	0.43
Plant fresh weight (g)	43.00	35.60	36.10	37.40	2.71
Root fresh weight (g)	14.35	13.88	15.58	21.10	3.05
No. of flowers	5.50	5.00	4.75	5.25	NS
Flower diameter (cm)	9.13	7.00	6.50	5.75	0.62
Flower pedicel length (cm)	30.80	14.80	14.50	7.33	2.50

LSD = Least significant differences; NS = Not significant

Table 2
Effect of Cultar used as soil drench on growth and flowering of *Rudbeckia laciniata*

Characters	Treatments			
	Control	1.0 mg	2.0 mg	$LSD_{0.05}$
Plant height (cm)	94.00	71.50	63.25	10.76
No. of branches	5.50	4.75	4.75	NS
No. of nodes	5.50	5.25	5.25	NS
No. of leaves	3.75	4.00	4.25	NS
Leaf fresh weight (g)	1.73	1.74	1.89	0.12
Plant fresh weight (g)	43.00	35.20	40.67	3.26
Root fresh weight (g)	14.35	15.90	19.60	2.06
No. of flowers	5.50	4.75	4.00	NS
Flower diameter (cm)	9.13	8.50	8.50	NS
Flower pedicel length (cm)	30.80	23.10	22.80	5.56

LSD = Least significant differences; NS = Not significant

As shown in Tables 1 and 2, plant fresh weight was reduced when using Cultar with either method compared to the control plants. The decrement percentages were 17.2, 16.0 and 13.0% at 250, 500 and 1000 ppm, respectively. Soil drench applications led to reductions of 18.14% at 1.0 mg and 5.42% at 2.0 mg. Root fresh weight was significantly increased at high concentrations of Paclobutrazol added as foliar spray (Table 1) or as soil drench (Table 2). The increment percentages were 23% with foliar spray at 1000 ppm and 47% with soil drench at 2.0 mg compared to control plants.

Control **250ppm** **500ppm** **1000ppm**

Fig. 1. Effect of Cultar used as foliar spray on the height of *Rudbeckia laciniata*

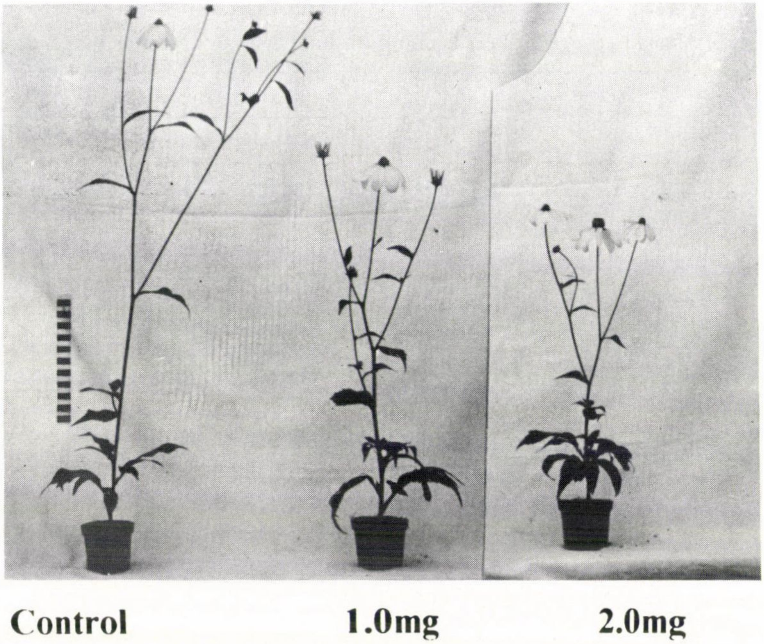

Fig. 2. Effect of Cultar used as soil drench on the height of *Rudbeckia laciniata*

With respect to the flower characteristics (Tables 1 and 2), Cultar had an only insignificant effect on flower number. Flower diameter was significantly decreased by spraying Cultar. The effect of soil drench applications was insignificant. Flower pedicel length was reduced by Cultar. Plants sprayed with Cultar were shorter than those treated with soil drench and both were significantly shorter than the control. The shortest pedicel length was obtained after the plants were sprayed at 1000 ppm.

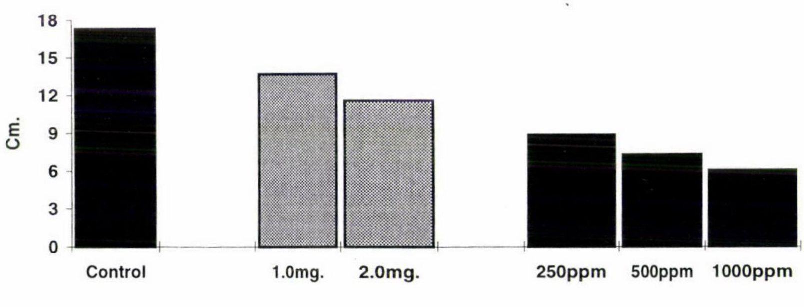

Fig. 3. Effect of Cultar used as foliar spray or as soil drench on the length of the internode

Discussion

As indicated by the results, Paclobutrazol decreased the plant height of *Rudbeckia* plants when applied at increasing concentrations. These results were in agreement with those reported by Deneke and Keever (1992) for tulips, Mao et al. (1991) for scarlet sage (*Salvia splendens* Buchoz ex Etl) and Hagiladi and Watad (1992) for *Cordyline terminalis* L., who found that Paclobutrazol was effective in reducing plant height, with a significant negative correlation between application rate and plant height. This decrement was due to a shortening of internode length not to the number of nodes per plant. Similar results were found by Le Cain (1986) for weeping fig (*Ficus benjamina* L.) and Suh et al. (1991) for tulips.

The number of branches per plant was not affected by Paclobutrazol at the concentrations used as soil drench or as foliar spray. Similar results were found by Holcomb and Rose (1992) for Poinsettias (*Euphorbia pulcherrima* Willd. Ex Klotzsh) and Song et al. (1991) for native Korean plants including *Chrysanthemum indicum* L.

Paclobutrazol had no significant effect on the leaf number of *Rudbeckia* plants. Hagiladi and Watad (1992) reported that the number of leaves was not affected by Paclobutrazol, except at the highest drench concentrations.

Plant fresh weight was reduced when using Cultar. These results were in agreement with those found by Wang and Gregg (1994) for golden pothos (*Epipremnum aureum* Lind. et Andre).

Cultar used at high concentrations increased the root fresh weight of *Rudbeckia*. Ruter (1992) found that the root:shoot ratio was higher in Paclobutrazol-treated plants than in the untreated control.

The number of flowers per plant was not affected by Cultar. Phetpradap et al. (1994) stated that the flower number were not affected by plant growth regulators at the concentrations used. Similar results were found by John and Ruter (1992) for the butterfly bush (*Buddleja davidii* Franch.). Flower diameter was only reduced by foliar spray application. This agreed with the findings of Gary et al. (1988) for marigolds (*Tagetes erecta* L.). Flower pedicel length was shorter after the plants were treated with Cultar compared to that of untreated plants (Ruter, 1992).

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ISOLATION AND CHARACTERIZATION OF PROMOTER TRAP TAGGED MUTANTS OF *ARABIDOPSIS THALIANA* EXHIBITING AN INCREASED SENSITIVITY TO FREEZING STRESS

J. ISAKSSON[1], E. MÄNTYLÄ[3], B. WELIN[3], K. LINDSEY[2] and A. MANDAL[3]*

[1]DEPARTMENT OF CELL RESEARCH, UPPSALA GENETIC CENTER, SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES, BOX 7055, S-750 07 UPPSALA, SWEDEN
[2]DEPARTMENT OF BIOLOGICAL SCIENCES, UNIVERSITY OF DURHAM, DURHAM DH1 3LE, UK
[3]DEPARTMENT OF MOLECULAR GENETICS, UPPSALA GENETIC CENTER, SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES, BOX 7010, S-750 07 UPPSALA, SWEDEN
*AUTHOR FOR CORRESPONDENCE.

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Promoter trap T-DNA tagging and *in vivo* gene fusion were employed for the isolation of specific genes of *Arabidopsis thaliana* that might be involved in the tolerance of plants against abiotic environmental stress, e.g. freezing and drought. Screening of the T_2 progeny of 2500 primary transformants by histochemical assay resulted in the isolation of two T-DNA tagged lines (Nos 274 and 317) that exhibit a low-temperature-responsive enhanced expression of the promoterless *gus* reporter gene. In these lines, GUS activity is also induced by the exposure of plants to abscisic acid (ABA). The pattern of altered gene expression in these lines was previously characterized based on the quantitative assay of GUS activity and northern analysis of the corresponding mRNA transcripts (Mandal et al., 1995). Our recent results revealed that acclimated plants of line 274 are sensitive to freezing stress; some individuals do not survive when exposed to –4°C overnight. The sensitivity of these plants to freezing stress was determined by assessing the degree of damage caused by leakage of the ions in the leaf tissues. T-DNA flanked plant sequences have been cloned by inverse PCR. Sequence analysis of the cloned plant DNA fragments is in progress.

Key words: *Arabidopsis thaliana*, gene fusion, low temperature, T-DNA tagging

Introduction

Abiotic environmental stresses, such as low temperature (LT) and drought, constitute a major constraint in crop distribution and productivity (Levitt, 1980). Until recently, attempts were being made to overcome this constraint mainly by altering the stressful environment to fit the plant. But such attempts are either impossible, economically prohibitive, or ecologically unsound. Therefore, emphasis has been shifted to modification of the plant to fit the environment by introducing genetically engineered plants with new and improved characteristics, e.g. stress tolerance or disease resistance.

During the past decade several molecular techniques have been employed for the identification and cloning of specific stress-responsive plant genes and there has been considerable progress in studies of the molecular genetic basis of the plant stress tolerance. Until now, several such genes that respond to cold, drought and salt stress have been cloned from a number of tolerant plant species and characterized (Guy, 1990; Palva, 1994) and they seem to fall in a few distinct categories that are ubiquitous in different plants (Nordin et al., 1991; Lâng and Palva, 1992; Nordin et al., 1993).

For the identification of LT- or ABA (abscisic acid)-responsive genes that might play a role in tolerance of plants against both freezing and drought stress a library of 2500 T-DNA tagged *Arabidopsis thaliana* plants has been screened. Two transgenic lines that exhibit an altered expression of the *gus* reporter gene in response to these stimuli have been identified. The pattern of altered gene expression in these lines (Nos 274 and 317) has been reported previously (Mandal et al., 1995). The present paper reports recent results on the further characterization of the T_3 progeny of these T-DNA tagged lines. Besides LT- and ABA-induced GUS activity and the accumulation of mRNA, plants of line 274 exhibit a mutant phenotype showing increased sensitivity to freezing stress.

Materials and methods

Plant materials and production of T-DNA tagged lines

For the identification of *in vivo* gene fusions in *A. thaliana* (ecotype C24) two T-DNA tagging vectors were employed as reported previously (Mandal et al., 1995). Root explants isolated from 3- to 4-week-old axenically grown *A. thaliana* were used for T-DNA mediated transformation. Transformation procedures, tissue culture conditions, the selection of transgenic lines and the *in vitro* production of transgenic seeds were essentially as described by Mandal et al. (1993).

Identification of in vivo gene fusions

In vivo gene fusions in the T-DNA tagged lines of *A. thaliana* were identified based on histochemical staining of the leaf tissues (Jefferson, 1987). For the identification of inducible gene fusions the plants were exposed to LT or exogenous ABA. The exposure procedures were essentially the same as described previously (Mandal et al., 1995) but with the following modifications: four-week-old plants grown in sterile conditions on solidified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) were exposed to either 4°C or liquid MS medium supplemented with 0.5 mg/l ABA for two days using a 16h photoperiod per day (45 $\mu E\ m^{-2}\ s^{-1}$) and analysed again by histochemical staining. Induced gene fusions were estimated based on a comparison of the activity of the *gus* reporter gene under constitutive (uninduced) and induced conditions. Plants exhibiting an altered GUS activity in response to these treatments were further characterized by fluorometric assay (Jefferson, 1987). For the determination of the kinetics of altered gene expression the plants were exposed to LT or ABA for 0, 2, 4, 8, 16, 24 and 48h and screened for the accumulation of the reaction product methylumbelliferone (MU) in the leaf tissues. Furthermore, to confirm that the induced GUS activity was indeed caused by altered gene expression, these plants were also characterized at the transcriptional level by northern analysis. The isolation and purification of the total plant RNA as well as the hybridization experiments were essentially as described previously (Mandal et al., 1995).

Determination of the number of T-DNA copies

Seeds collected from the T_1 progeny were germinated for three weeks under sterile conditions on MS medium supplemented with 20 g/l sucrose and 50 mg/l kanamycin. The copy number of the integrated T-DNA was estimated based on the segregation pattern of the T_2 progeny, i.e. the ratio of kanamycin-resistant (km^R) to kanamycin-sensitive (km^S) seedlings. The results of this analysis were verified essentially by Southern analysis (Southern, 1975).

Identification of stress-sensitive mutants

For the identification of a mutant phenotype, the four-week-old axenically grown kanamycin-resistant T_2 plants were first exposed to either 4°C for one week or exogenous ABA (liquid MS medium supplemented with 0.5 mg/l ABA) for two days in order to induce stress hardiness, also known as acclimation. During these treatments a 16h photoperiod per day (45μE m^{-2} s^{-1}) was maintained. The plants were then transferred to –4°C overnight for the identification of frost-sensitive individuals. T_3 progeny of some selected individuals exhibiting a strong induction of *gus* reporter gene by LT or ABA and sensitivity to freezing stress were investigated further to estimate the rate of frost-induced injuries. The freezing sensitivity of soil-grown T-DNA tagged plants of line 274 was determined by exposing the excised rosette leaves to subzero temperatures in a controlled temperature bath (Lâng et al., 1989). The degree of injury in the leaf tissues caused by freezing temperatures was determined by measuring the ion leakage from the tissue (Sukumaran and Weiser, 1972). In all experiments wild-type *A. thaliana* plants (ecotype C24) were treated similarly and used as controls.

Results and discussion

This paper is part of a series of studies on the identification, isolation and characterization of a specific class of plant genes that might play a role in the tolerance of plants against environmental stress such as frost, drought, salinity, insect injuries and pathogen infection. The model system employed for the tagging and cloning of such specific genes is *Agrobacterium tumefaciens* T-DNA-mediated *in vivo* gene fusion in *Arabidopsis thaliana*. Up till now, 2500 T-DNA tagged lines have been generated by employing two promoter trap vectors as described previously (Mandal et al., 1995). For the identification of *in vivo* fusions of the *gus* reporter gene to constitutively expressed or inducible genes of *A. thaliana* all these transgenic lines have been screened by histochemical staining (Jefferson, 1987). The frequency of gene fusion to constitutively expressed genes based on GUS^+ phenotypes was observed to be 20% (496/2500) on average in the lines tested (data not shown). These results are in agreement with those reported previously (Mandal et al., 1995).

For the identification of LT- or ABA-related gene fusions the transgenic lines were exposed to LT and exogenous ABA and screened again for induced GUS activity. So far, two transgenic lines (Nos 274 and 317) have been isolated that exhibit an induced expression of *gus* reporter gene in response to both LT and exogenous ABA. Results on the preliminary characterization of the pattern of altered gene expression in these two lines were reported previously (Mandal et al., 1995).

The next question was whether any stress-related mutant phenotype was associated with these plants. If the integrated T-DNA had indeed disrupted a significant LT- or ABA-induced gene or regulatory sequence that could function as promoter, a mutant phenotype could be expected, particularly an increased sensitivity to freezing and/or drought stress. Acclimated plants of lines 274 and 317 (pre-exposure of plants to LT for induction of stress hardiness) did not exhibit any phenotypic changes in comparison with the control plants in response to drought stress (data not shown). However, some individuals of line 274 showed severe damage when exposed to freezing stress; they could not survive exposure to –4°C overnight (Fig. 1).

Fig. 1. Identification of frost-sensitive mutants of *A. thaliana*. T_2 plants were grown axenically for 3 to 4 weeks on selective MS medium supplemented with 20 g/l of sucrose and 50 mg/l of kanamycin. Kanamycin-resistant plants were first exposed to LT (4°C) for 7 days for the induction of stress hardening. They were placed in a climate chamber at –4°C overnight and then transferred to normal growth conditions (22°C, 50% relative humidity). Two individual plants of line 274 (on the left) could not survive freezing stress. The photograph was taken three days after the regrowth of the stressed plants

For the determination of the sensitivity of these plants to freezing stress excised leaves of non-acclimated plants of line 274 were subjected to controlled freezing and the injury was measured at different subzero temperatures. Traditionally, 50% ion leakage is considered lethal. Our results show that ice formation caused severe injury to plants of line 274 even at temperatures where the control plants were not affected (Fig. 2). These results suggest that line 274 has reduced ability to tolerate or control ice formation in the plant tissue at subzero temperatures.

To characterize the kinetics of the LT-dependent altered expression of the *gus* reporter gene in more detail the plants of line 274 were exposed to LT for 0 to 48 h and screened by fluorometric analysis (quantification of the GUS activity during induction) as described by Jefferson (1987). The results of this analysis are presented in Table 1. Table 1 indicates that a twofold induction in the GUS activity could be detected after a 2-hour exposure of plants to LT and this activity rose by a factor of at least 8 when exposed for 24 hours. These results are in agreement with those obtained during preliminary characterization and reported previously (Mandal et al., 1995). The comparatively quick induction (2h) of the *gus* reporter gene suggests that the putative T-DNA tagged plant promoter or regulatory elements respond actively from the beginning of exposure of plants to LT, leading to an early activation of the plant's stress tolerance machinery.

Table 1
Quantitative analysis of GUS activity in T-DNA tagged *Arabidopsis thaliana*

Plants tested[a]	GUS activity[b] in pmole methylumbelliferone/mg protein/min after a 0–48h exposure of plants to low temperature							
	0	2	4	8	16	24	48	IF[c]
274	0.13	0.25	0.67	0.81	0.92	1.14	0.32	8.8
VEC	0.05	0.05	0.06	0.05	0.06	0.06	0.05	1.2
CON	0.05	0.05	0.05	0.05	0.04	0.06	0.06	1.2

a T-DNA tagged line 274 was transformed with pMHA2 (Mandal et al., 1995). VEC and CON represent the vector-transformed (pDE1001, Denecke et al., 1992) and untransformed *A. thaliana* plants, respectively used as controls. Plants were grown as described in the legend to Fig. 1 and exposed to LT (4°C) for 0, 2, 4, 8, 16, 24 and 48h prior to quantitative GUS analysis. [b]Quantitative analysis of GUS activity was performed as described by Jefferson (1987). [c]Induction factor (IF) is the ratio between GUS activity in plants with (24 h) and without (0 h) exposure to LT.

In conclusion, a T-DNA tagged mutant of *A. thaliana* has been isolated that exhibits an induced expression of the *gus* reporter gene in response to low temperature or exogenous abscisic acid. Some individuals of this line, when exposed to freezing stress, exhibit severe damage, leading to the death of the plants. These results provide evidence that a specific sequence of *A. thaliana* has been tagged that can function to promote LT- or ABA-induced gene expression.

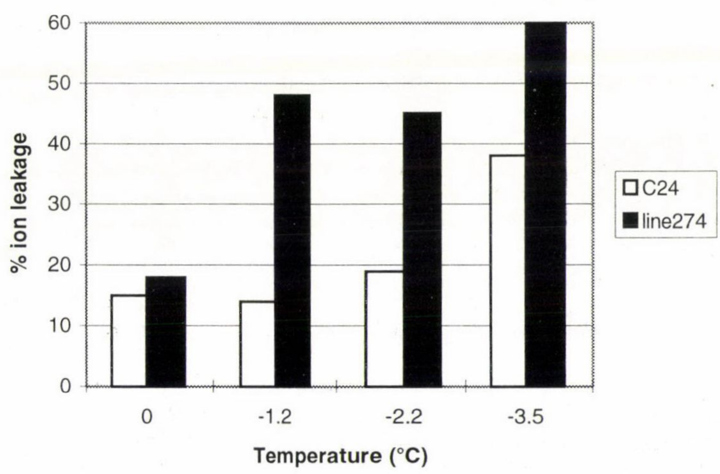

Fig. 2. Frost injury of excised leaves of greenhouse-grown C24 and line 274 plants. Leaves of soil-grown plants were exposed to freezing temperatures in a controlled temperature bath. The temperature was lowered 2°C/h and samples were removed at desired temperatures. Relative ion leakage from the leaves (injury) was estimated by measuring conductivity after exposure to the chosen temperature and subsequently after freezing the tissue in liquid nitrogen (100% ion leakage)

This sequence may belong to plant promoters or their regulatory elements that may or may not correspond to functional genes. So far, there is no genetic or molecular evidence to prove that a functional gene or promoter is "tagged" with a T-DNA insertion. The data obtained from segregation analysis of the T_2 progeny under selective conditions and later verified by Southern blot hybridization indicate that there are two copies of the integrated T-DNA in line 274. Recently, two plant DNA fragments have been cloned from this line flanking upstream of the promoterless *gus* reporter gene by inverse PCR. The size of these fragments is in agreement with those obtained in Southern analysis (data not shown). From each fragment approximately 300 bp have been sequenced. Further sequencing is in progress.

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IDENTIFICATION OF QTL INFLUENCING FREEZING TOLERANCE IN BARLEY

R. TUBEROSA[1], G. GALIBA[2], M. C. SANGUINETI[1], E. NOLI[1] and J. SUTKA[2]

[1] DEPARTMENT OF AGRONOMY, VIA FILIPPO RE 6, UNIVERSITY OF BOLOGNA, ITALY
[2] AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, H–2462 MARTONVÁSÁR, P.O. BOX 19, HUNGARY

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Significant genetic variation for freezing tolerance under controlled conditions at –12°C and –14°C was detected among 64 doubled-haploid lines derived from the cross Arda × Opale. Based on a 672 cM-long map, the number of QTL (quantitative trait loci) detected at –12°C and –14°C was equal to four and six, respectively. The phenotypic variation accounted for by these QTL was limited, most likely due to the fact that portions of the genome remain unexplored.

Key words: barley (*Hordeum vulgare* L.), freezing tolerance, QTL

Introduction

Tolerance to freezing temperatures is an important component of winterhardiness, a key trait for the release of barley cultivars better adapted to a wide range of environments. The complex nature of freezing tolerance has hindered progress in the improvement of this trait. Molecular markers not only provide a powerful tool for identifying the chromosomal regions harbouring the QTL (quantitative trait loci) controlling winterhardiness but allow for the verification of the role which specific biochemical characteristics (e.g. cold-induced proteins, membrane composition, abscisic acid accumulation, etc.) may have on freezing tolerance. In barley, extensive work carried out by Hayes and coworkers (Hayes et al., 1993; Pan et al., 1994; Oziel et al., 1996) has led to the identification on chr. 7 of a multilocus cluster of QTL regulating winter-hardiness. The comparison of QTL position in different genetic backgrounds and/or environments could allow for the identification of QTL characterized by large stable effects which would thus represent good candidates for cloning procedures. In this work, we have utilized molecular markers and doubled-haploid (DH) lines to investigate the genetic basis of freezing tolerance in a barley cross.

Materials and methods

Plant material

Sixty-four DH lines were randomly chosen from a set of 72 DH lines obtained by Noli et al. (1994) from a cross between Arda and Opale (two- and six-rowed winter cultivars, respectively) in order to identify QTL for abscisic acid (ABA) concentration in the leaf (Noli et al., 1996). Previous field observations showed that Opale is more winterhardy than Arda (unpublished results).

Evaluation of freezing tolerance

Freezing tolerance was assessed in the phytotron facilities of the Agricultural Research Institute in Martonvásár (Tischner et al., 1997). Seed was germinated in Petri dishes in the dark at 15°C. Seedlings (five/line/frost treatment in four consecutive replicates) were then subjected to gradually decreasing temperatures as follows (day and night temperatures, respectively): 15°C/10°C (1st week), 10°C/5°C (2nd and 3rd week), 5°C/0°C (4th and 5th week). Afterwards, the temperature was reduced by 1°C/h until it reached –4°C. Hardening was continued for 48 h in the dark, after which freezing treatment was applied for 24 h at –12°C or –14°C. The temperature was then raised by 2°C/h to 1°C; afterwards, the plants were kept at 1°C for 15 h. The plants were then recovered at 16°C (day) and 15°C (night) for 18 days and were evaluated for survival.

Molecular marker analysis

The molecular analysis of the DH lines was based on 149 markers (RAPDs, RFLPs and STSs) using procedures described in Noli et al. (1997). RAPD markers were obtained using primer sets from Operon. The code names of the RAPDs include the letters OP (Operon), the kit code, the primer identification code and estimated amplicon size expressed in base pairs (e.g. OPAL13-650).

Statistical analysis

Analysis of variance (ANOVA) and QTL analysis were both performed on the arcsin of the rate of freezing survival (ARFS) at –12°C and –14°C and their mean values. QTL analysis was carried out with the computer programme QGENE (Nelson, 1997) kindly provided by C. Nelson, Cornell University, NY. The genetic map was obtained using Mapmaker (Lander et al., 1987) with the Kosambi function, an LOD of 2.5 and a maximum recombination of 37.5%.

Results and discussion

Molecular analysis

In total, 116 RAPDs, 25 RFLPs and 8 STSs were utilized to generate a 121-point linkage map. For 28 RAPDs there was coincidence of map position in groups of two or more markers. Groups of coincident RAPD bands occurred more frequently in regions near the centromere. The markers were resolved in 12 linkage groups for a total of 672 cM. Ten linkage groups were attributed to the seven chromosomes based on the information available for the RFLP and STS markers. The average distance of RFLP markers was approx. 30% shorter than that evidenced for the same markers in previously published maps (Langridge et al., 1995).

ANOVA

The ANOVA evidenced significant differences between the DH lines with values ranging from 0 to 100% (non-transformed data; Table 1) at –12°C and from 0 to 93.8% at –14°C. The combined ANOVA showed highly significant ($P<0.01$) effects due to freezing treatments (72.9% at –12°C and 59.8% at –14°C) and to DH lines, whereas the "DH line × freezing treatment" interaction was not significant. Heritability values computed on a mean DH line basis were high and equal to 0.86 (–12°C), 0.80 (–14°C) and 0.90 (mean). The parental lines did not differ significantly; the decrease in survival rate from –12°C to –14°C was more marked in Opale (from 95% to 47.5%) than in Arda (from 81.7% to 65.0%). Transgressive values of DH lines were observed in both directions.

Table 1

Mean survival rate (%) of the doubled-haploid (DH) lines, F test between DH lines and heritability values on a mean basis

Treatment	Mean	Min.	Max.	F	h^2
–12°C	72.9	0.0	100.0	**	0.86
–14°C	59.8	0.0	93.8	**	0.80
Mean	66.3	0.0	95.0	**	0.90

** P<0.01

QTL analysis

Table 2 reports the main parameters of the QTL. Given the P value herein considered (P=0.05), it is likely that one or more of these QTL may represent statistical artifacts. The number of significant QTL (underlined in Table 2) was equal to four at –12°C and six at –14°C. Different sets of QTL were evidenced at the two temperatures, thus indicating that the role of the genetic factors influencing freezing survival is related to the severity of the stress. We have included one putative QTL on chr. 2 which was associated to marker OPG13-650 with a P value slightly above the 0.05 threshold at –12°C (P=0.0582) and at –14°C (P=0.0504); the P value of the same marker was 0.0466 when the analysis was carried out on the means. Additionally, at this QTL the magnitude and the direction of the additive effect (a) was similar in the two treatments. It is noteworthy that for the remaining QTL too the effects of the parental alleles were consistent in the two treatments. At –14°C, three of the six QTL were localized on chromosome 6 within an interval of approx. 50 cM. A similar result was also reported by Hayes et al. (1993) for a region on chromosome 7 controlling winterhardiness; the same authors have indicated the possible causes of such findings. Two QTL (on chr. 3 and chr. 7) which were identified at –12°C were also evidenced when the analysis was carried out on the mean values of the two temperatures; analogous results characterized two QTL (on chr. 3 and chr. 6) at –14°C and in the mean.

Table 2
Parameters of QTL for arcsin rate of freezing survival (ARFS) of DH lines. Underlined values refer to QTL significant at $P<0.05$

			–12°C		–14°C		Mean	
Marker	Chr.	Source[1]	$R^{2(2)}$	$a^{(3)}$	$R^{2(2)}$	$a^{(3)}$	$R^{2(2)}$	$a^{(3)}$
OPG13-650	2	Arda	9.4	0.13	9.9	0.11	10.3	0.12
OPAM13-1200	2	Arda	0.3	0.02	8.6	0.11	3.1	0.06
OPG16-670	3	Opale	11.9	–0.14	4.9	–0.08	8.9	–0.11
OPG13-680	3	Arda	4.6	0.08	8.0	0.10	6.6	0.09
MWG514	6	Arda	7.6	0.13	3.1	0.07	5.7	0.10
OPAC14-1400	6	Arda	3.5	0.07	7.0	0.09	5.4	0.08
OPG02-1100	6	Arda	3.7	0.08	8.3	0.10	6.1	0.09
ABG458	6	Arda	5.2	0.09	8.3	0.10	7.2	0.10
OPG04-1230	7	Opale	8.3	–0.11	4.6	–0.07	7.0	–0.09
Multilocus			22.3		21.1		25.3	

(1) Parent contributing the allele which increased ARFS value
(2) Percentage of phenotypic variance explained
(3) Additive effect of the allele at the marker locus computed as: (Arda - Opale)/2

The amount of phenotypic variation for ARFS between DH lines accounted for by each of the significant QTL varied from 6.6% to 11.9%. The alleles increasing freezing tolerance were contributed in equal number by the parents at –12°C while at –14°C only the Arda alleles showed favourable effects. Multilocus regression analysis was carried out including only significant QTL (underlined in Table 2). Despite the high values of the heritability estimates (see Table 1) the R^2 values were low and equal to 22.3% at –12°C, 21.1% at –14°C and 25.3% in the mean. This finding is most likely related to the fact that a sizeable portion of the genetic map remains unexplored. In order to fill the gaps in our map, we are currently adding AFLP and SSR markers. The low multilocus R^2 values could also be due to i) an asymmetric distribution of the frequencies of the allelic combinations possible for the significant markers, as a consequence of the small population size, and/or ii) epistatic interactions among the QTL. The comparison of our results with those of Hayes et al. (1993) indicates that the QTL near OPG04-1230 maps in a position corresponding to that of the QTL on chr. 7 evidenced in their cross (Dicktoo × Morex). The effect observed at this QTL in Dicktoo × Morex was much larger than that observed in our population. The above-mentioned findings only show circumstantial evidence for the presence of possibly orthologous QTL in the two populations, it should be pointed out that the Dicktoo × Morex population was tested in the field while our evaluation was carried out at the seedling stage in growth chambers. Therefore, in our case, only freezing tolerance was evaluated but no other components of winterhardiness such as vernalization requirement, growth habit and photoperiod response. Additionally, Dicktoo and Morex showed differences in winter survival much larger than those observed between Arda and Opale. Of the nine QTL influencing ARFS, four mapped in regions which previous work on the same population has shown to contain QTL affecting the ABA concentration of the flag leaf in drought-stressed field conditions near anthesis (Noli et al., 1996). In wheat, ABA concentration in the crown tissue has been shown to play an important role in freezing tolerance (Gagne et al., 1989). Additionally, it is worth mentioning that the QTL on chr. 7 evidenced by us and in the work of Hayes et al. (1993) maps in a region containing an ABA-responsive gene (*Dh-1*) coding for a dehydrin. Further work is necessary to elucidate the possible role of these factors in regulating the response to freezing tolerance in barley.

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PLEIOTROPIC EFFECTS OF DWARFING *(RHT)* GENES IN NEAR-ISOGENIC LINES OF COMMON WHEAT CV. BEZOSTAYA IN A CENTRAL EUROPEAN ENVIRONMENT

D. MIAZGA, A. J. WORLAND[1] and K. KOWALCZYK

INSTITUTE OF GENETICS AND PLANT BREEDING, AGRICULTURAL ACADEMY, LUBLIN, POLAND
[1]JOHN INNES CENTRE, NORWICH, ENGLAND

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The effects of dwarfing genes *Rht1, Rht1S, Rht1K* and *Rht12* in near-isogenic lines of common wheat cv. Bezostaya were studied. In all the experimental years the lines with dwarfing genes were significantly shorter than the controls (rht). Reduction in height was in the order: *Rht1S* < *Rht1* < *Rht1K* < *Rht12*. Moreover, a significantly lower 1000-grain-weight was found in the *Rht12* lines in each year of the study, whereas isogenic lines with genes *Rht1S, Rht1* and *Rht1K* had significantly lower values only in the 3rd year. These annual differences point to the influence of environmental conditions and may be linked to interactions between temperature and fertility.

Key words: *Triticum aestivum,* semi-dwarfness, reduced height, *Rht* genes

Introduction

In recent decades significant increases in wheat production have occurred worldwide mainly due to the introduction of varieties carrying semi-dwarfing genes (Gale and Youssefian, 1985).

The majority of the world's semi-dwarf wheat varieties carry one of the two homoeologous dwarfing genes *Rht1* or *Rht2*, both of which are derived from the old Japanese variety Norin 10. *Rht1* and *Rht2* appear to be very similar genes, both being able to combine height reductions of around 20% with yield increases of up to 20% under appropriate environmental conditions. Increases in yield are obtained by the dwarfing genes promoting a significant increase in spikelet fertility. Both *Rht1* and *Rht2* can be detected and selected in segregating populations by their insensitivity to exogenous gibberellic acid (GA_3) (Gale and Youssefian, 1985). The ability to detect *Rht1* and *Rht2* in segregating populations permitted their backcrossing into a number of standard varietal backgrounds to develop near-isogenic lines to fully assess their pleiotropic effects on a range of agronomic characters. Studies by Worland (1987) and Kertész et al. (1991) suggest that both *Rht1* and *Rht2* might be environmentally sensitive and show decreased spikelet fertility when subjected to temperatures of above 24°C during the period from flag leaf to ear emergence. Environmental susceptibility will limit the spread of the dwarfing genes in new varieties to areas where they are not likely to encounter over-high temperatures during critical growth stages. Winter wheat varieties carrying *Rht1* or *Rht2* are uncommom in several Central European countries, such as Germany, Poland and Switzerland (Worland, 1986).

Alternative dwarfing genes are, however, available that might offer greater environmental adaptability to Central European countries like Poland. These include *Rht1S, Rht1K* and *Rht12*.

Rht1S is an allelic variant of *Rht1* located at the same position on chromosome 4B (nomenclature: 7th International Wheat Genetic Symposium, Cambridge, 1988). Although *Rh1S* was originally derived from the old Japanese variety 'Saitama 27' it has been successfully utilised in Italian wheat varieties since 1947 (Worland, 1986) and has since spread to Yugoslavian, Hungarian and Romanian wheat varieties grown in areas where *Rht1* or *Rht2* would be ill-adapted (Worland, 1986; Jošt, 1989; Kertész et al., 1991). *Rht1S* is characterised by a weaker insensitivity to GA_3 than *Rht1* and only reduces height by about 10%. In many Southern European wheats the moderate height reduction of *Rht1S* is combined with the height-reducing effects of *Rht8* or *Ppd1* to produce a more adapted semi-dwarf phenotype.

Rht1K is yet another allelic variant of *Rht1* but is in this instance a variant that is associated with a decrease in height reduction of around 30%. Again the height reduction is correlated with increased spikelet fertility (Börner et al., 1995; Worland et al., 1995). *Rht1K* was derived from a natural mutant of the Russian variety 'Bezostaya 1' known as 'Krasznodari 1' or 'Bezostaya Dwarf' mutant (Börner et al., 1995; Worland et al., 1995). *Rht1K* has shown selective advantages in Central Europe, being present in many Russian varieties, and in Hungarian varieties derived from 'Mv 13' (Worland, 1986).

A contrasting dwarfing gene, *Rht12,* was derived in Hungary as an X-ray mutant of the variety 'Karcag' (Viglasi, 1968). *Rht12* is sensitive to GA_3 so cannot be selected using the GA test in segregating populations. *Rht12* is, however, extremely potent, reducing height by over 50% and is dominant, so lines carrying *Rht12* are easily distinguished in segregating populations. *Rht12* is located on chromosome 5A (Sutka and Kovács, 1987) and shows no recombination with awn inhibitor b1. This means that when the *Rht12* gene is introduced into awnless Western European wheat varieties that normally carry the b1 awn inhibitor, the presence of awns in the b1, b1 homozygous genotypes implies the presence of the dominant *Rht12* gene and can be used as a selection marker in the development of isogenic lines.

In order to study the pleiotropic effects of *Rht1, Rht1S*, *Rht1K* and *Rht12* and to determine which genes were most suitable for use in the development of semi-dwarf wheats adapted to the Polish environment, isogenic lines were developed by backcrossing each gene into a 'Bezostaya 1' background. 'Bezostaya 1' was chosen as the recipient parent as this variety has proven environmental adaptability to Polish and Eastern European environments.

Materials and methods

Genetic stocks

Near-isogenic lines for the dwarfing genes *Rht1, Rht1S, Rht1K* and *Rht12* were developed by backcrossing donor lines carrying the different dwarfing genes with the quality Russian variety 'Bezostaya 1'. At least six backcrosses were carried out, and heterozygous backcross progeny carrying the dwarfing genes were selected between the backcrosses, either by utilising the gibberellic acid (GA_3) screening test described by Gale and Gregory (1977) or for *Rht12* by selecting for the dominant dwarf phenotype. After the completion of backcrossing, homozygous dwarf lines and homozygous tall controls for the GA_3-insensitive dwarfing genes *Rht1, Rht1S* and *Rht1K* were again selected using the GA test, whilst homozygous dwarfs for *Rht12* were selected on the basis of short stature and awned (b1, b1) phenotype, with the recessive awn inhibitor b1 showing complete linkage to *Rht12* (Worland et al., 1995).

Experimental design

Experiments were carried out over 3 seasons (1992/93, 1993/94 and 1994/95) at Czes³awice, Poland. In year 1 five replicates were hand-sown, with each genotype represented by a row of 11 seeds spaced 10 cm within rows and 30 cm between rows. In year 2 each of the four replicates comprised about 1000 germinated kernels hand-sown in 2 m^2 plots. In the final year 550 germinated kernels per m^2 were machine-sown in 10 m^2 plots. For each plot the heading date was recorded as the number of days from 1st May until full flowering. Height was measured on 4 random plants. In the first year 4 ears per plot were removed prior to plot harvest for the evaluation of number of spikelets/spike, number of grains in the 1st and 2nd florets and in the whole spike, weight of grains/spike, 1000 grain weight and spikelet fertility. In the 2nd and 3rd years twenty spikes were removed from the larger plots for similar character evaluation.

The results obtained were statistically analysed individually for each year. The ANOVA programme was used, applying the F-Snedecora test. In order to find the significance of the differences between isogenic lines, Tukey's intervals of confidence were used.

Results and discussion

Table 1 presents the mean values of the analysed quantitative traits in the near-isogenic lines of var. 'Bezostaya'. In all the experimental years lines with dwarfing genes were significantly shorter than the controls (rht). Reduction in height was in the order: *Rht1S* < *Rht1* < *Rht1K* < *Rht12* as shown in Germany by Börner et al. (1995). In the Polish studies, *Rht1S* lines were characterised by a height reduction of about 17% in relation to the tall control, whereas Worland (1987), Worland and Petrović (1988) and Worland et al. (1990) showed *Rht1S* to reduce height in the U.K. by about 11%. A larger reduction in height of just over 20% was observed in *Rht1* lines, agreeing with the *Rht1* height reduction in other genetic backgrounds (Kertész et al., 1991; Börner et al., 1993; Miazga et al., 1993). Reduction in height in the isogenic lines of var.'Bezostaya' with *Rht1K* genes was over 25%. This again agrees with the results of Börner et al. (1995) in research conducted in Gatersleben and with those of Worland et al. (1995) for experiments grown in England. The reduction of plant height in the *Rht12* lines was the highest, being above 50%, as observed by Konzak (1982) and Worland et al. (1994).

Table 1

Pleiotropic effects of *Rht* genes on yield and its components in near-isogenic lines of Bezostaya (Czesławice, Poland, 1992–95)

Character	Bezostaya *rht*			Bezostaya *Rht1S*			Bezostaya *Rht1*			Bezostaya *Rht1K*			Bezostaya *Rht12*		
	1993	1994	1995	1993	1994	1995	1993	1994	1995	1993	1994	1995	1993	1994	1995
Plant height (cm)	80.3	111.4	112.8	70.8**	91.6**	91.1**	63.6**	83.3**	83.1**	55.2**	75.1**	74.4**	34.7**	49.2**	53.7**
Ear emergence (days)	30.0	35.0	33.5	31.0	34.8	33.0	31.0	34.8	34.0	31.0	35.0	33.5	33.0**	38.0**	38.0**
No. of kernels in ear	39.8	45.6	37.3	41.2	45.1	35.9	43.6	44.1	34.5	35.3	48.8*	34.1	36.2	43.0	30.4
No. of kernels in 1st and 2nd florets	29.0	31.6	27.4	32.8	31.1	25.6	32.6	30.4	26.6	29.7	30.8	28.1	28.0	30.2	28.2
Fertility of spikelets	2.34	2.43	2.06	2.33	2.56	2.26	2.48	2.58	2.26	2.16	2.77	2.0	2.33	2.46	1.87
Fertility of 1st and 2nd florets	1.71	1.68	1.52	1.85**	1.76	1.61	1.86**	1.77	1.54	1.82	1.75	1.65	1.80	1.73	1.74
1000 grain weight (g)	55.9	56.2	52.5	55.1	51.4	40.8**	51.6	47.8	39.1**	51.3	45.3	37.2**	45.4**	37.2**	34.8**
Weight of grains in ear	2.22	2.56	1.96	2.27	2.32	1.46	2.25	2.10	1.35**	1.81**	2.20	1.27**	1.64**	1.61**	1.05**
Harvest index (%)	42.8	37.8	38.2	47.7**	43.3**	43.5**	47.7**	42.3*	42.6*	46.6*	42.7**	42.5*	51.9**	35.0*	38.2

* = Significant differences between the original variety and the *Rht* lines at the 5% level
** = Significant differences between the original variety and the *Rht* lines at the 1% level

The number of days from the 1st of May to the heading date in the controls (rht) was 30.0, 35.0 and 33.5, respectively, in the consecutive experimental years. A similar number of days was observed in the *Rht1S, Rht1* and *Rht1K* lines, indicating, as found previously in Eastern Poland by Miazga et al. (1993), that GA-insensitive dwarfing genes do not alter flowering time. The date of heading for *Rht12* lines in all the experimental years was significantly later than in the control (Table 1), as previously found by Worland et al. (1995).

In the present study the number of kernels in the ear and in the 1st and 2nd florets was variable, and depended on the year. In 1993 lines *Rht1S* and *Rht1* formed more kernels (41.2 and 43.6, respectively) than the rht controls (39.8), whereas in the remaining two years the control lines set slightly more kernels per spike and in the 1st and 2nd florets than the *Rht1S and Rht1* isogenic lines. The *Rht1K* line only set more seed per ear than the controls in 1994. Slightly fewer kernels were set in the ear and in the 1st and 2nd florets of *Rht12* lines compared to the controls in all the years (Table 1). Previously Gale and Youssefian (1993), Allan (1989) and Borrell et al. (1991) showed that *Rht1* increased fertility and kernels per ear. Kertész et al. (1991), Börner et al. (1993) and Miazga et al. (1993), however, found more variable results depending on the *Rht1* gene, the year and the variety, but generally found that *Rht1* lines showed a higher number of kernels in the ear than the controls. Similarly, Worland (1989) and Worland and Petrović (1988) showed that varieties with *Rht1S* set a significantly higher number of kernels in the ears, whilst Börner et al. (1995), using near-isogenic lines in var. 'Bezostaya' and 'Cappelle-Desprez', found that in Germany *Rht1S* increased the number of seeds per ear in Silstedt and Rieste but produced no significant changes at Hohenthurm. The same authors found very similar results for *Rht1K,* with again increases in grains per ear at all sites except Hohenthurm.

Spikelet fertility in the near-isogenic lines studied in Czeslawice was close to or higher than in the controls (Table 1). In the individual years the spikelet fertility of *Rht1* lines was 6.0 to 9.7% higher than that of the controls. The highest spikelet fertility (2.77) was observed in the *Rht1K* line in the second year. This value was 14% above that of the controls. Miazga et al. (1993) previously observed a differentiated spikelet fertility under Polish conditions in lines of 'Maris Huntsman' and 'Maris Widgeon' carrying *Rht1* genes depending on the year and the variety. In the U.K. Gale and Youssefian (1985) found a significantly higher fertility in all *Rht1* lines, suggesting that the *Rht1* allele is better adapted to the U.K. than to the Polish climate. This could well be due to climatic interactions occurring around the time of meiosis, as Worland and Law (1985) previously showed that temperatures above 24°C at this critical growth phase reduced spikelet fertility. Whilst such temperatures are rare in the U.K. in late May, in Poland they are more common, preventing the *Rht1* lines from obtaining maximum spikelet fertility. The near-isogenic lines were also characterised by a higher fertility in the 1st and 2nd florets than the controls in all the experimental years. Differences were significantly higher in 1993 for lines *Rht1* and *Rht1S*.

Previously Worland (1987) and Worland and Petrović (1988) found that the fertility of the 1st and 2nd florets was higher in *Rht1S* forms, but the differences were not significant. In Eastern Poland the differences found by Miazga et al. (1993) for isogenic lines of 'Maris Huntsman' and 'Maris Widgeon' were only significant in some seasons.

Gale and Youssefian (1983) found that in the U.K. *Rht1* lines regularly produced higher spike grain weight than the *rht* controls. In the present Polish experiment the *Rht1* and *Rht1S* lines only had higher spike grain weight in 1993, while a significantly lower grain weight was found in 1995 in the ears of *Rht1* isogenic lines and in 1993 and 1995 in the *Rht1K* isogenic lines. This could again be the result of higher temperatures reducing ear fertility in Poland. The GA-responsive isogenic line *Rht12* reduced spike yield in Poland in all the experimental years. Similar results were found in the U.K. by Worland et al. (1995). The reduction in yield was probably due to *Rht12* delaying flowering.

In the three years a lower 1000 grain weight was observed in all dwarf isogenic lines (Table 1). Only in 1993 was the thousand grain weight of the 'Bezostaya' *Rht1S* line (55.1 g) close to that of the control (55.9 g). In all the years a significantly lower 1000 grain weight was also found in the *Rht12* lines, whereas isogenic lines with *Rht1S, Rht1* and *Rht1K* genes were only significantly lower in the 3rd year of the study. These annual differences point to the influence of environmental conditions and are probably linked to interactions between temperature and fertility. Previous studies by Gale and Youssefian (1983) also indicated that dwarf isogenic lines had lower 1000 grain weights in the U.K., the reduced 1000 grain weight being a reflection of increased spikelet fertility.

In each of the years the harvest indexes of *Rht1S, Rht1* and *Rht1K* were significantly higher than for the rht control (Table 1). For *Rht12* lines the 1993 harvest index was significantly higher and the 1994 index significantly lower, with no difference in 1995. Previously in Poland Miazga et al. (1993) studied isogenic lines with *Rht1, Rht2, Rht1+2, Rht3* and *Rht2+3* alleles in 'Maris Huntsman' and 'Maris Widgeon' backgrounds and demonstrated a similar increase in harvest index for *Rht1* lines, reflecting the reduction in straw weight in semi-dwarf lines.

Conclusions

1. In all the experimental years lines with dwarfing genes were significantly shorter than the controls (rht). Reduction in height was in the order: *Rht1S* <*Rht1*< *Rht1K* <*Rht12*.
2. The date of heading for *Rht12* lines in all the years was significantly later than in the control.

3. In the present study the number of kernels in the ear and in the 1st and 2nd florets was variable, and depended on the year and the lines.
4. In all the years a significantly lower 1000 grain weight was found in the *Rht12* lines, whereas isogenic lines with *Rht1S, Rht1* and *Rht1K* genes had significantly lower values only in the 3rd year of the study. These annual differences point to the influence of environmental conditions and may be linked to interactions between temperature and fertility.

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COMPARATIVE STUDY OF THE MINERAL COMPOSITION OF RED CLAYS IN HUNGARY

J. FEKETE, P. STEFANOVITS and G. BIDLÓ*

GÖDÖLLŐ UNIVERSITY OF AGRICULTURAL SCIENCES, DEPARTMENT OF SOIL SCIENCE AND AGRICULTURAL CHEMISTRY, H–2103 GÖDÖLLŐ, HUNGARY
*TECHNICAL UNIVERSITY, DEPARTMENT OF ENGINEERING GEOLOGY, H–1521 BUDAPEST, HUNGARY

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Red clays in Hungary are the products of soil forming processes in earlier geological times. They are generally situated in areas that were mainlands in the Tertiary Period and so were not covered by marine sediments. The climate of the Quaternary Period eroded them and today they can only be found in areas where they were protected against degradation in the Pleistocene or, due to their resistance and thickness, the material was able to withstand erosion. Red clays are therefore fossille or relic products of soil formation. Since both their water regime and nutrient supply differ from soils formed in the Holocene, their economic importance is far from negligible; vineyards, forests and arable lands can be found on these areas.

Key words: red clay, red soil, fossil soil, relic soil, tropical weathering, laterization, rubefication, bauxite formation

Introduction

Different views on the formation, properties and distribution of red soils in Hungary have been published by several authors. Geologists took a stand on the origin of red clays. Lóczy (1886) described the red clay material as a variety of loess, a rock formed by the deposition of wind-blown silt material. Loess formed under drier conditions and red clay under more humid climatic conditions, though the age and origin of red clay and loess are identical. Treitz (1903; 1912) shared this opinion, stating that red clay, as a product of soil-forming processes, was formed from wind-blown material in the Quaternary Period. It is the B horizon of soils under forests established on loess, where the original A horizon was eroded. Timkó and Ballenegger (1915) had similar ideas about these soils. A peculiar type of red clay is the clay soil formed on rocks of volcanic origin (nyirok) in Tokaj-Hegyalja (foothills of the Tokaj mountain), which was first described by Szabó (1866) and characterized in detail by Ballenegger (1917), who reinforced Szabó's ideas. The clay soil (nyirok) at Tokaj-Hegyalja is a relic from the Tertiary Period formed by the weathering of young, volcanic rocks and their tuffs from the Tertiary Period under subtropical climatic conditions. The term "nyirok", in a wider sense, is often used for red clays in general.

The contemporary knowledge on the conditions and characteristics of red clay, and of red and yellow soil formation in Hungary and in other countries was summarised by 'Sigmond (1934). Sümeghy (1944; 1949) analysed the earlier ideas about red clays and "nyirok" in detail. In his opinion, red clays and yellow materials differ from each other only in colour, iron content and contamination. Their major characteristic features are that they do not contain lime, they are sticky and soft, are liable to swelling and rapid drying, and they contain iron compounds. Red clays are rocks featuring unique characteristics, and are not to be confused with other types of rock.

According to Vendl (1957) red clays can be found in the depressions of areas covered with massive limestone and dolomite. As the area rose, the clay material moved into the depressions of the limestone with the rainfall. Under the milder, Mediterranean climate, iron-containing compounds in the clays became oxidized, and the iron oxides formed give the red colour of the clay.

Different ideas have developed about the distribution and characteristics of red clays and loamy products (Ötvös, 1954; Vadász, 1956; Bidló, 1974; Borsy and Szöőr, 1981; Jámbor, 1980; Jánossy, 1979; Kretzoi, 1969; Pécsi, 1985; Schweitzer, 1993). Stefanovits (1959; 1963) discovered that the red clays of Hungary are genetically diverse. Soils formed on sandstone can also be considered as belonging to the red clays (Vendl, 1957; Vadász, 1960; Bulla, 1962; Juhász, 1987). Some authors draw parallels between red clay formation and the process of bauxite formation, or consider bauxitic formations to be red clays (Vadász, 1956; Vendl, 1957).

Kubiena (1956; 1958) studied the formation of red clays thoroughly. In his opinion, red clay soils are the products of different processes. The two main processes of formation he called laterization and rubefication. While laterization is associated with the mobilization of silicic acid and the simultaneous accumulation of aluminium and iron compounds, rubefication is the process when iron hydrous oxides coagulate within a short time after the dissolution of iron from primary minerals. He explains the difference at the micromorphological level. The jelly-like feature of "rotlehm" is characteristic of the lateritic formations, while rubefication produces a flocculent, structured effect. The latter is characteristic of the "terra rossa" formations, and soils in the temperate zone.

Bárdossy (1989; 1990) also distinguished between bauxite, bauxitic clay and terra rossa. He considered bauxite to be the product of soil formation as well, which could develop in one place or be the result of redeposition.

The FAO World Soil Map also differentiates between red soils. As indicated by Driessen and Dudal (1991), Plinthosols and Ferralsols can be characterized by a great amount of mobilisable iron and aluminium compounds, and the similarly red Cambisols (Chromic Cambisols) by relatively moderate weathering.

There is a fundamental difference between these two directions of soil formation with regard to the clay mineral composition as well. While Plinthosols and Ferralsols primarily contain a kaolinite-based clay mineral association, the presence of illite is revealed in Cambisols (Fekete, 1981).

Based on the relevant literature, there are, therefore, major differences between both the conditions of formation and the characteristics of red soils and red clays.

Changes in climatic conditions, plate tectonics and crust movements make these facts even more complex. It is therefore not certain that red clays were formed as far from the Equator as they are situated now.

Materials and methods

The samples for the examination of red clays were collected from 61 soil profiles all over the country. Among these, results from the analyses of 8 red clay samples will be presented in this paper. Samples were selected to represent the more important sites of occurrence and types of red clays.

Sampling sites of red clay samples:

108. Mád	40–60 cm
2. Aggtelek – I.	7–20 cm
100. Jósvafő	20–55 cm
65. Vörösberény	20–44 cm
68. Balatonalmádi	80–100 cm
75. Máriagyűd	100–130 cm
120. Kakasd	60–80 cm
88. Nagygombos	115–130 cm

The Mád sample was collected in a soil profile next to the zeolite mine called Subalyuk, SW of Mád. This sample is representative of red clays formed from rhyolite and rhyolite tuff in the Zemplén mountains.

The Aggtelek – I sample is from a soil profile on the edge of a dolina between the village of Aggtelek and Vöröstó (Red Pond) NW of the highway. The Jósvafő sample comes from a soil profile in the valley of the Tohonya Creek. Data of these two profiles are typical of red clays in the Northern Borsod karst region.

The sample from Vörösberény (20–44 cm) represents the bauxitic formations in the Transdanubian mountain range, while the sample from Balatonalmádi (80–100 cm) is characteristic of the soils formed on Permian red sandstone.

Red clays of the Transdanubian hilly region are characterized by the data of two samples. These are from the fissure of a limestone mine north of Máriagyűd in the Mecsek and Tolna-Baranya hilly regions, and from a site SW of Kakasd.

On the northern and western borders of the Great Hungarian Plain red clays can often be found in exposures. In many places the red clay or loamy layers extend under the surface. The data of the Nagygombos sample represent these territories.

Basic soil science and mechanical analyses were performed on the red clay samples.

X-ray diffraction and (derivatographic) thermal analysis were applied to determine the mineral composition. [Equipment used for X-ray diffraction analysis: computer-controlled Philips diffractometer; PW generator; controllable PW 1050 goniometer; Philips Analytical PC-APD Diffraction software. X-ray tube: Cu anode with LFT monochromator, range of angle 2Θ–5–70°. Thermal analysis was done with a Paulik-Paulik-Erdey type derivatograph: MOM derivatograph, temperature region: 20–1000 °C, sensitivity of the TG: 100 and 200 mg.]

Results

The data of the basic analysis are shown in Table 1, and those of the mechanical composition in Table 2. Derivatograms are presented in Figures 1–8. The data on mineral composition obtained from X-ray diffraction and thermal analysis are summarised in Table 3.

Table 1
Data of basic analysis of red clay samples

Sample and number			K_A	hy_1	pH KCl	pH H_2O	$CaCO_3$ %	Humus %
108	Mád	40 – 60 cm	41	5.73	6.12	6.78	–	0.92
2	Aggtelek – I.	7 – 20 cm	64	3.62	6.74	6.89	0.12	0.61
100	Jósvafő	20 – 55 cm	62	9.72	5.91	6.75	–	0.19
65	Vörösberény	20 – 44 cm	60	3.33	7.59	8.15	5.80	3.29
68	Balatonalmádi	80 – 100 cm	46	1.31	7.11	7.37	0.12	1.31
75	Máriagyűd	100 – 130 cm	42	1.85	7.89	8.50	39.77	–
120	Kakasd	60 – 80 cm	51	3.73	7.47	7.74	–	0.18
88	Nagygombos	115 – 130 cm	54	6.56	8.00	8.14	1.32	0.22

The texture of the Mád (40–60 cm) sample is loam, with a clay content of 41% and a <0.01 mm fraction of 59%. The sticky point according to Arany is 41, but the hygroscopic value of 5.73% indicates clay texture. The reason for this is that the clay fraction contains a relatively large amount of smectite-type clay minerals. This sample contains only a few percent of kaolinite (Table 3). The mineral composition is characterised by an approximately 30% quartz content, and the fine fraction by a 13% feldspar content. Among the clay minerals, the illite content of the original sample is 32.8%, while the montmorillonite content of the fine fraction is 42.8%. Neither goethite nor hematite was detected (Fig. 1, Table 3), so the red colour of the clay (5YR 4/6) must originate from its amorphous iron content.

Table 2
Mechanical composition of red clays as percentages

Sample and number		>0.25 mm	0.25–0.05 mm	0.05–0.01 mm	0.01–0.005 mm	0.005–0.001 mm	<0.001 mm	>0.01 mm	<0.01 mm
108 Mád	40–60 cm	0.87	8.71	31.07	2.00	12.20	45.15	40.65	59.35
2 Aggtelek – I	7–20 cm	0.26	0.33	7.42	3.95	8.06	79.98	8.01	91.99
100 Jósvafõ	20–55 cm	0.64	0.00	20.17	6.76	13.29	59.14	20.81	79.19
65 Vörösberény	20–44 cm	3.41	9.85	19.09	5.65	21.91	40.09	32.35	67.65
68 Balatonalmádi	80–100 cm	2.74	34.74	21.28	6.33	11.35	23.56	58.76	41.24
75 Máriagyûd	100–130 cm	5.36	34.37	7.35	7.35	11.21	34.36	47.08	52.92
120 Kakasd	60–80 cm	1.17	41.10	5.48	1.57	3.89	46.79	47.75	52.25
88 Nagygombos	115–130 cm	5.22	12.58	13.78	4.31	8.52	55.59	31.58	68.42

Table 3
Mineral composition of the red clay samples as percentages

Minerals	Sample and number															
	Mád		Aggtelek – I.		Jósvafő		Vörösberény		Balatonalmádi		Máriagyűd		Kakasd		Nagygombos	
	108		2		100		65		68		75		120		88	
	original	f.f.*	original	f.f.	original	f.f.	original	f.f.	original	f.f.	original	f.f.	original	f.f.	original	f.f.
Quartz	32.3	28.1	3.3	4.1	59.5	37.2	3.4	31.8	27.6	39.8	16.9	15.6	29.8	13	43.2	28.6
Calcite	–	1.3	–	–	2.7	–	5.4	3.6	–	–	23.6	21.2	1.1	–	2.7	1.5
Dolomite	–	–	–	–	–	–	–	–	–	–	14.5	8.8	–	–	–	–
Feldspars	1.5	13.1	–	–	2.0	2.0	–	–	–	–	6.2	32.0	10.2	6.3	–	–
Kaolinite	2.0	8.1	68.0	65.0	28.5	14.9	35.6	32.3	20.9	25.3	25.2	1.8	26.0	19.4	4.8	10.9
Chlorite	–	–	–	–	–	–	–	–	–		–	–	10.6	4.0	–	–
Illite or muscovite	32.8	–	–	–	–	–	–	–	47.1	4.6	6.8	2.4	–	–	–	–
Montmorillonite + amorphous	25.0	42.8	–	–	–	39.0	14.4	–		20.0	–	12.7	16.4	48.9	42.3	50.0
Boehmite	–	–	–	–			–	16.1	–	–	–	–	–	–	–	–
Gibbsite	–	–	–	–			1.8	5.4	–	–	4.2	2.2	–	–	–	–
Hematite	–	–	2.1	7.9	0.8	0.5	5.0	8.0	2.3	6.3	–	–	–	0.8	–	–
Goethite	–	–	20.0	19.0	–	–	–	–	–	–	–	–	–	–	–	–
Humus	1.4	1.4	2.2	2.0	2.1	2.0	4.0	2.8	0.8	1.4	0.4	1.6	0.8	1.0	0.4	0.8
H_2O^-	4.4	4.0	2.8	2.0	3.2	3.6	2.0	3.8	1.0	1.8	1.8	1.3	4.7	5.4	6.0	7.0
H_2O^+	0.6	1.2	0.6		1.2	0.8	–	–	0.3	0.8	0.4	0.4	0.4	1.2	0.6	1.2

* fine fraction

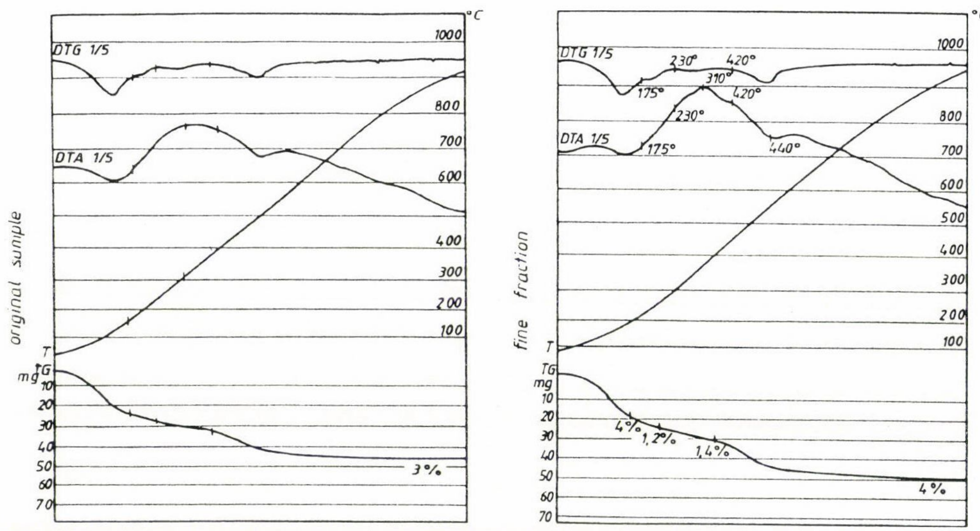

Fig. 1. Derivatogram of the Mád (40–60 cm) sample, No. 108

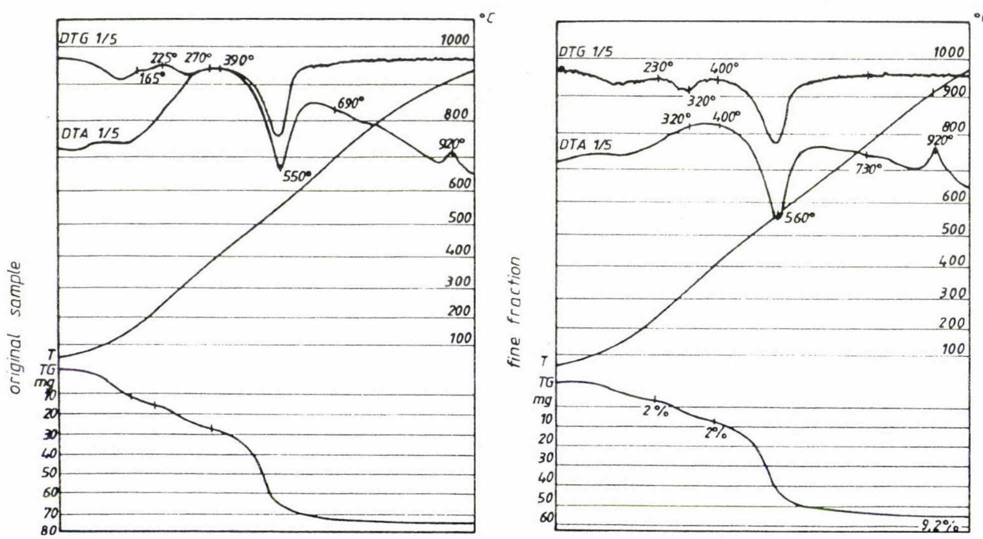

Fig. 2. Derivatogram of the Aggtelek-I (7–20 cm) sample, No. 2

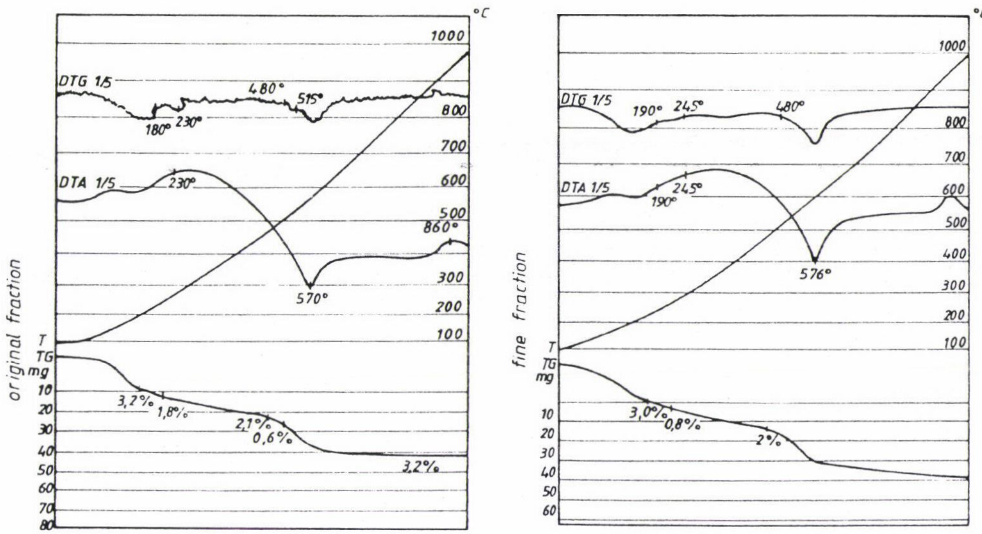

Fig. 3. Derivatogram of the Jósvafő (20–55 cm) sample, No. 100

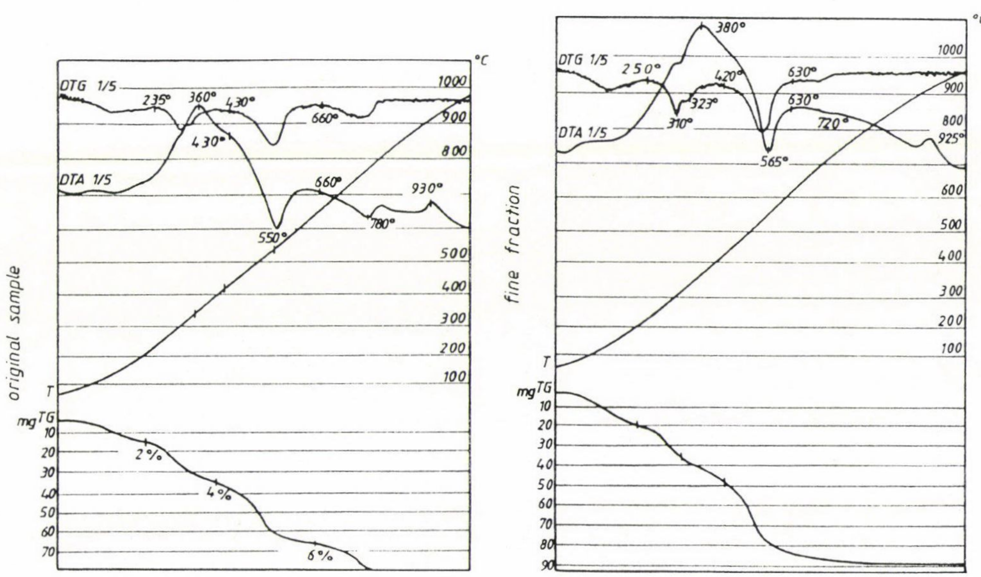

Fig. 4. Derivatogram of the Vörösberény (20–44 cm) sample, No. 65

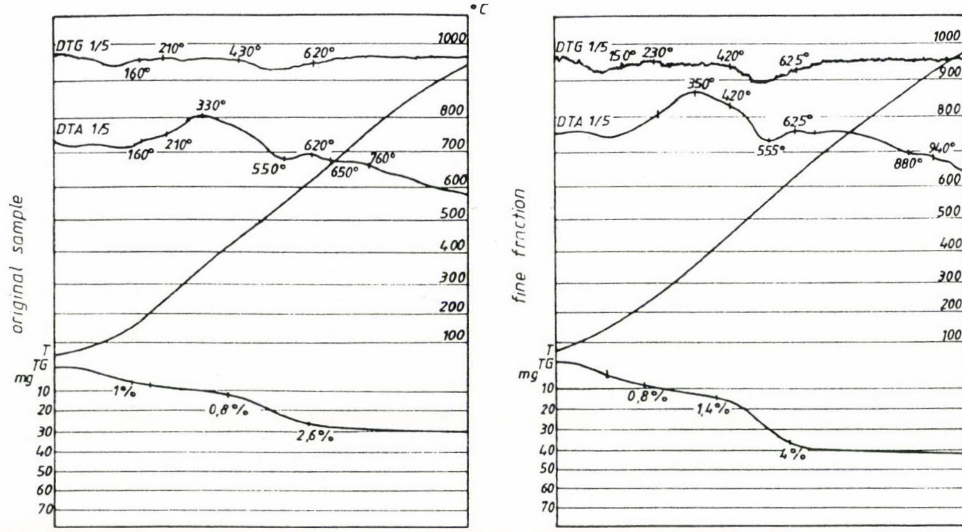

Fig. 5. Derivatogram of the Balatonalmádi (80–100 cm) sample, No. 68

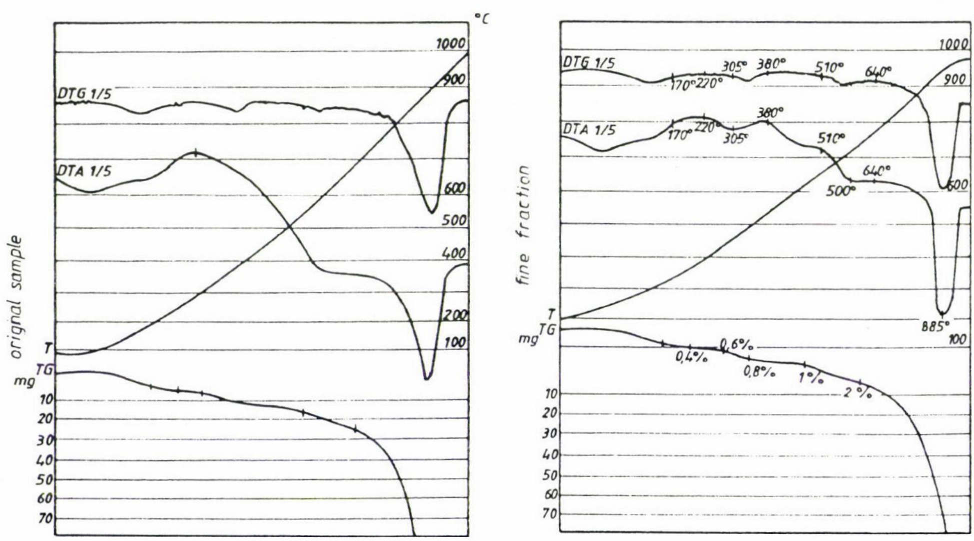

Fig. 6. Derivatogram of the Máriagyűd (100–130 cm) sample, No. 75

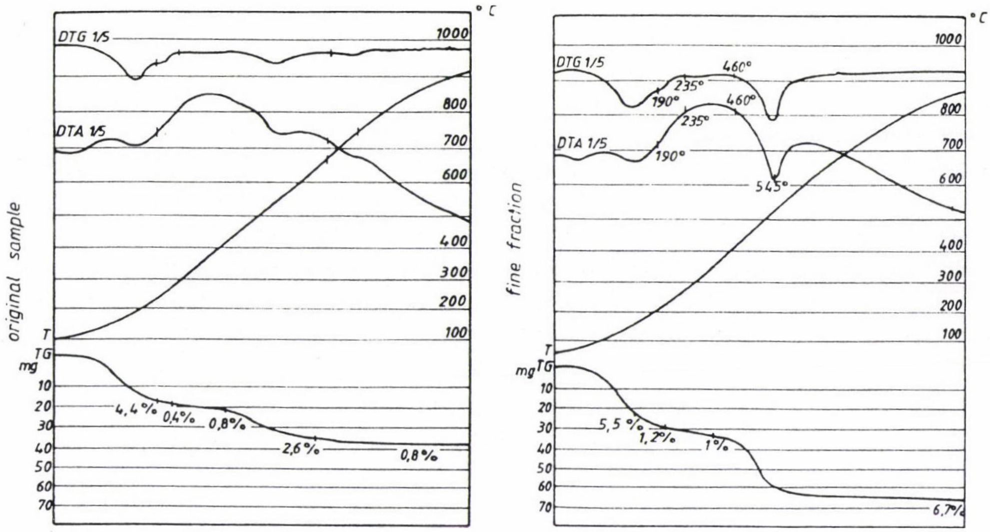

Fig. 7. Derivatogram of the Kakasd (60–80 cm) sample, No. 120

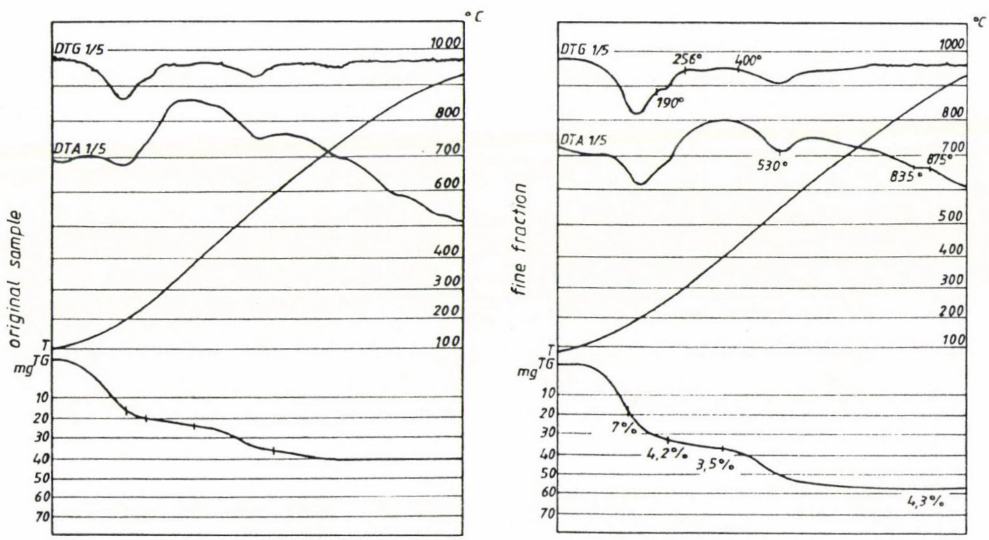

Fig. 8. Derivatogram of the Nagygombos (115–130 cm) sample, No. 88

The red clay examined was formed *in situ* from the underlying rhyolite tuff. This is supported by the fact that the particles of the sand fraction are not rounded, but angular. Its unfavourable properties are that it does not contain calcium carbonate and has only a low humus content. In the foothills of the Tokaj mountain, around the settlements of Ond, Szegilong, Tolcsva and Sárospatak, similar red clay formations are present, though in some of these places the clay content of the red soil is higher than in the Mád sample.
Red soils are relatively frequent in the Northern Borsod karst region, but they differ in colour, hardness and in other properties. The differences are also evident in the physical properties and mineral composition of the two selected samples. The clay content of the Aggtelek-I sample is very high, 80%, and the <0.01 mm fraction content is 92%. The clay content of the sample from Jósvafő is 60%, and the <0.01 mm fraction content is also lower, 79%. The sticky point according to Arany is high in both samples, 64 and 62, respectively, notwithstanding the hygroscopic moisture content, which shows a significant difference between the two samples, with values of 3.62 and 9.72, respectively. The 3.62% value found for the Aggtelek-I sample is extremely low compared to its very high clay content. This can be explained by the different clay mineral compositions. The clay fraction of the Aggtelek-I sample contains around 65% kaolinite. The kaolinite content of the Jósvafő sample is much lower, in addition to which it contains montmorillonite, which reaches 39% in the fine fraction. The mineral composition of the two samples are well illustrated by the different curves of the derivatograms. Quartz is only present in the Aggtelek-I sample in trace amounts, there is little hematite, and the goethite content is relatively high, around 20%. The Jósvafő sample contains a lot of quartz and only a few feldspars; among the iron oxides only hematite is present in a small amount. The iron compounds that are responsible for the red colour are present possibly in amorphous form. In spite of the fact that the parent material of these soils is limestone, they contain practically no calcium carbonate and their humus content is also very low. These red clays were formed *in situ* on Mesozoic limestone. Loess formation probably had an impact on the genesis of the red clay in Jósvafő. The red clay from the Tertiary Period became mixed with falling material later. This idea seems to be supported by the higher loess content (20%), and the different quartz content and mineral composition of the Jósvafő sample.

The Vörösberény (20–44 cm) sample is representative of bauxitic derivatives. Their colour is brown-red (10R 4/6) in the upper layers and dark brown-red (2.5YR 3/6) in the lower layers. The clay fraction is 40%, with a <0.001 mm fraction of 67.6%, so the texture is clay loam. The sticky point according to Arany is higher than would be expected from the clay content. Among the clay minerals, kaolinite dominates, with 32% in the fine fraction. This is the reason for the relatively low hygroscopic value. The quartz content of the fine fraction is 31.8%; it also contains 3–5% calcite, 16% boehmite, 5.4%

gibbsite and 8% hematite, which are characteristic of bauxitic formations. The presence of kaolinite indicates tropical weathering products as well. The bauxite was formed partly at the end and partly at the beginning of the Cretaceous Period. The red clay of the severely eroded upper layers of bauxitic areas are relics of tropical and subtropical soils from the Tertiary Period.

The Balatonalmádi (80–100 cm) sample was collected from a soil formed on Permian red sandstone. Its clay content is around 24% and the <0.01 mm fraction is also only 41%, so its texture is loam. The sticky point according to Arany is 46, which is typical of clay loams; its hygroscopic value is 1.31, which is characteristic of sandy loam soils. The quartz content of the fine fraction is around 40%. Among the clay minerals, kaolinite, illite, montmorillonite and chlorite can be detected in the sample. Hematite represents the iron-containing minerals in the sample.

The peculiar soils of the Balaton highlands were formed on Permian sandstone. According to Bulla (1962), the oldest soil formations in Hungary are the redeposited soil materials that have been conserved in the bright red, sometimes purplish, Permian sandstones. The soil became mixed with marine sediments and formed a rock. Naturally, the soil formed on the Permian bedrock and lying on it today is not a Paleozoic relic, but originates from the end of the Tertiary period and inherited the red soil material. The red soils of vineyards and gardens in Révfülöp, Szepezd and Csopak are such formations.

The Máriagyűd (100–130 cm) sample was collected in one of the fissures of a limestone mine NW of the village. It represents the red soils of the Southern Baranya hilly region and the Villányi limestone mountains. Its clay content is 34%, the <0.01 mm fraction is around 53%, the sticky point according to Arany is 42, and the hygroscopic moisture content is 2%, so its texture is considered to be loam. The quartz content is over 15%, and besides 20% calcite, it also contains 10% dolomite. Its feldspar content is significant. Among the clay minerals, kaolinite, montmorillonite and illite are present. Its aluminium-containing mineral is gibbsite; there is no iron-containing mineral present; the red colour originates from amorphous iron compounds.

The fissure-caves and funnels in the Mecsek mountains have often been covered with similar red soils.

Stefanovits (1967) claimed that the red soils on the limestone of the Baranya inselbergs were terra-rossa formations, formed under Mediterranean conditions.

The Kakasd (60–80 cm) sample represents the red soils of the Szekszárd hilly region. Its texture belongs to the loam category, it contains a relatively small amount of clay and the total amount of the clay and silt fractions is only 52%. It contains relatively little quartz and calcite. Its feldspar content is 10 and 6%. Among the clay minerals, its kaolinite and montmorillonite contents are significant, but chlorite is also present in the sample. Among the iron oxides, only hematite can be detected in small amounts.

The red clay formation of the Transdanubian hilly region can be dated from the Pliocene Period (Ádám, 1969; Schweitzer, 1993). Their formation was the result of the weathering of the Pannonian surface from the end of the Miocene until the lower Pleistocene. They are extensive in the Tolna hilly region, especially in the Zselic area, and traces of them can be found in the Outer-Somogy region.

The Nagygombos (115–130 cm) sample comes from below a sandy soil with low organic matter content. Similar red soil can be found close to the surface in the wall of a nearby mine belonging to the brick-works in Hatvan. The clay content of the Nagygombos sample is 56%, and the <0.01 mm fraction reaches 68%. The sticky point value according to Arany and the hygroscopic value reflect the texture. There is approximately 50% montmorillonite and 5–10% kaolinite in the sample. The peculiarity of this kaolinite is that it does not exhibit basic reflection during X-ray diffraction analysis. The presence of this "degraded kaolinite" (nomenclature: G. Bidló) has been noticed in soft red clays in other samples as well. Hematite and goethite were not detected.

Red clay formations are relatively frequent on the northern and western borders of the Great Hungarian Plain. Such formations are the red clays of the Northern mountain range, the Buda mountains, the Transdanubian mountain range and the border area of the Transdanubian hilly region. These are soils that were formed in the Pliocene, and at the turn of the Pliocene and Pleistocene, and paleosols that were formed in the Pleistocene. There are clay, silt and sand layers of different origins lying under red soils on the borders of the Great Hungarian Plain. The lower layers of Hungarian loess exposures frequently hide red clay soils and red loamy soils (Pécsi, 1967). These red soils are the products of soil formation under strongly Mediterranean climatic conditions in the interglacial periods.

Summary

The red clays of Hungary are the products of soil-forming processes in earlier geological times. Their range of occurrence coincides with the location of tropical and subtropical mainlands in the Tertiary Period. Today they are covered by forests, or used for grape production and arable farming.

Hungarian red clays can be divided into several groups.

1. *Red clays of the foothills of the Tokaj mountains.* The <0.01 mm particle size fraction makes up about 60% of these soils. Besides quartz they contain feldspars, illite, montmorillonite and a small amount of kaolinite. They were formed on rhyolite or rhyolite tuff, and are covered by loess in some areas. They are relic soils, older than loess, formed under the warm climate of the Tertiary Period.

2. *Red soils of the Northern Borsod karst region.* Very heavy soils with 70–80% clay content. The dominant clay mineral is kaolinite, but they contain a significant amount of smectite as well. Their hematite and goethite content varies within the area. They are Tertiary relic soils formed on Mesozoic limestone. Laterite and bauxite formations can be detected in some places. The frequency of their occurrence varies.
3. *Bauxitic formations of the Transdanubian mountain range.* In Hungary, bauxite formation is the result of tropical weathering of the surface of inland limestone and dolomite. However, the red clays in the upper layer of the eroded bauxitic areas are relics of tropical and subtropical soils. They are characterised by their kaolinite, gibbsite, boehmite and hematite content.
4. *Red soils formed on Permian sandstones in the Balaton highlands.* The signs of the oldest soil formation in Hungary can probably be found in the Permian red sandstones. Their colour is red, sporadically with a purple nuance. They are rocks formed from a mixture of sediments and tropical red soils. Naturally, the soil formed on the Permian bedrock is not a Paleozoic remnant of a soil, but is a soil relic and originates from the end of the Tertiary Period. It is characterised by its kaolinite, illite, montmorillonite and hematite contents.
5. *Red clays of the Transdanubian hilly region.*
   a) Red clays formed by the weathering of the Pannonian surface. These soils have a medium clay content and contain kaolinite, montmorillonite, chlorite and a small amount of hematite. They were formed between the end of the Miocene Period and the lower Pleistocene.
   b) Red clays of the Mecsek and Villányi mountains. These red clays can be found on the surface, in depressions and fissures in the limestone. The clay minerals which occur are kaolinite, montmorillonite and illite. Gibbsite may also be present and the iron content is amorphous. These are terra-rossa formations, formed under Mediterranean conditions.
6. *Soils of the border region of the Hungarian Great Plain.* These soils have a medium clay content, with large quantities of montmorillonite and a small amount of kaolinite. Their iron content is often amorphous. They are situated on clay, silt and sand layers of different origin or between loess depositions. They were formed in the Pliocene and at the turn of the Pliocene and Pleistocene.

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STUDIES ON THE PHYTOTOXICITY OF HERBICIDES IN MAIZE *(ZEA MAYS* L.*)* AS AFFECTED BY TEMPERATURE AND ANTIDOTES

Z. BERZSENYI, B. GYŐRFFY, T. ÁRENDÁS, P. BÓNIS and D. Q. LAP

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES
H-2462 MARTONVÁSÁR, HUNGARY

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In a series of experiments carried out in the Martonvásár phytotron it was found that the phytotoxicity of the acetanilide herbicides tested increased in the following order: propachlor > alachlor > metolachlor > acetochlor. In the field experiments their weed control efficacy exhibited an opposite tendency. The phytotoxic effect of different rates of butylate and EPTC was highly dependent on temperature. The addition of antidote reduced the influence of temperature on thiocarbamate injury. The average growth inhibition for inbred lines in phytotron experiments was 54% for acetochlor (23% with antidote) and 12% for butylate (8% with antidote).

Key words: chloroacetamide, herbicide, injury, maize, phytotron, thiocarbamate

Introduction

Considerable differences exist between the degrees of efficacy and selectivity of the major herbicides (chloroacetamides, thiocarbamates, sulphonylureas) used in the weed control of maize. Maize genotypes (hybrids, inbred lines) have been shown to have different tolerance to numerous herbicides, such as atrazine, simazine, butylate, EPTC, alachlor, metolachlor, nicosulphuron, primisulphuron and thifensulphuron (Wright and Rieck, 1973; Narsaiah and Harvey, 1977; Sagaral and Foy, 1982; Green and Ulrich, 1993).

In addition to the inherited sensitivity of the hybrids or lines, climatic factors (precipitation and temperature), herbicide-soil interactions and the production technology have a fundamental influence on herbicide selectivity (Mulder and Nalewaja, 1970; Burt and Akinsorotan, 1976). High temperatures were shown to increase EPTC and butylate toxicity to maize. The phytotoxic injury of acetanilides is significantly influenced by the quantity of precipitation. Our experimental data did not indicate that an increase in temperature from 13°C to 22°C had any significant effect on the phytotoxic injury to maize plants (Berzsenyi and Győrffy, 1989). The tolerance of maize to the herbicides chloroacetamide and thiocarbamate is substantially improved by safeners or antidotes (Pallos and Casida, 1978).

The aims of the experiments were:

(1) to compare the phytotoxicity and weed control efficacy of chloroacetamide herbicides,

(2) to examine the effects of different temperatures on EPTC and butylate selectivity,

(3) to study the sensitivity of maize hybrids and inbred lines to acetochlor and butylate herbicides with and without antidotes.

Materials and methods

The series of experiments was conducted in the Martonvásár phytotron in a PGV plant growth chamber (Tischner et al., 1997) and a temperature gradient chamber (Tischner and Veisz, 1996). In the PGV chamber the day temperature was set at 18°C and the night temperature at 13°C, while the relative humidity was 80%. On the gradient bench a linear temperature gradient was maintained between 16–26°C. In both growth chambers the length of the photoperiod was 16 hours and the intensity of illumination 30 klux.

The pots were filled with a 1:1 ratio of chernozem and sandy soil. Eighteen seeds per pot were planted in the homogeneous chambers and 12 in the gradient chambers, 2 cm deep. The herbicides were applied using a special American spray boom. The length of the experiments was 21 days in the PGV chambers and 16 days in the gradient chambers. Each experiment was set up at least twice and there were 4–6 replications within each experiment.

Measurements and observations were carried out for each plant and the data were averaged for the plants in each pot. The phytotoxic injury caused by the herbicides was assessed by scoring on the 14th and 21st day using a 1–5 scale (1=no injury, 5=lethal injury). At the end of the experiments, measurements were made on shoot height and the green mass of the shoots. The antidotes, marked AD-67 and TI-35, were used at a 10:1 (w/w) ratio.

The field experiments were set up on chernozem soil with forest residues in the experimental nursery of the institute. The soil had an organic matter of 3.2%, a pH of 7.2 and good supplies of nutrients. The experiments were set up in 4 replications in a randomized block design. The plot size was 25 m^2. The herbicide effect was determined by scoring on several occasions (% weed cover per species) and by measuring the weed mass prior to maize harvesting. The maize grain yield was recorded at a moisture content of 15%.

Results and discussion

1. Comparative studies on the phytotoxicity and weed control efficacy of chloroacetamide herbicides

Figure 1 shows the fresh weight and plant height of three-week-old maize plants as affected by the rate of acetanilide herbicides expressed as a percentage of the control. It was found that the phytotoxic effect of propachlor was very slight and did not increase within the dosage limits examined. On the other hand, with the herbicides alachlor, metolachlor and acetochlor a definite dosage effect could be observed in the extent of phytotoxic injury. At a rate of 1 l ha^{-1} the phytotoxic effect of alachlor and metolachlor is small, whereas acetochlor has a significant phytotoxic effect at this rate. Raising the rates of alachlor, metolachlor and acetochlor to 2, 4 and 8 l ha^{-1}, the phytotoxic effect increased in the order listed. Regarding the degree of phytotoxic injury, the phytotoxic effect of alachlor can be considered moderate. The curves expressing the phytotoxic effect of metolachlor and acetochlor were nearly parallel, but the phytotoxic effect of acetochlor exceeded that of metolachlor.

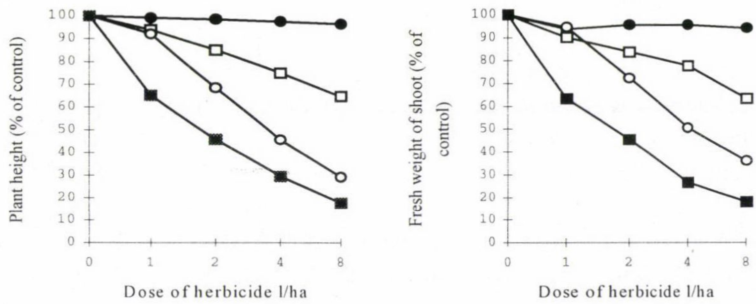

Fig. 1. Comparison of the phytotoxic effect of acetanilide herbicides as affected by the rate of application (● – propachlor, □ – alachlor, ○ – metholachlor, ■ – acetochlor)

The results of the field experiment (Table 1) show that the trend in the weed control efficacy of chloroacetamides was opposite to that of the phytotoxic damage. Propachlor had the poorest weed control effect, followed by metolachlor and alachlor, while acetochlor had a significantly better weed control effect. It can be seen from Table 1 that the herbicide effect increased for all the herbicides at greater rates, though the excellent herbicide effect of acetochlor was obvious even at the lowest (1 l ha^{-1}) rate.

Table 1

Comparative studies on the weed control efficacy and phytoxicity of chloroacetamide herbicides

Treatments	Rate l/ha^{-1}	Weed cover rate % 30 DAT	60 DAT	Green mass dkg m^{-2} Monocot	Dicot	Phytotoxicity 30 DAT	Grain yield t ha^{-1}
Hand weeded	–	–	–	–	–	–	9.5
Weedy control	–	60	84	122	35	–	2.0
Propachlor	1	14	45	48	48	2.3	5.0
Propachlor	2	12	38	28	33	2.5	5.8
Propachlor	4	6	24	7	28	2.8	8.5
Propachlor	8	8	25	3	19	2.8	8.6
Propachlor	16	4	12	4	9	3.3	9.4
Alachlor	1	11	48	34	28	2.2	6.5
Alachlor	2	11	44	9	36	2.8	7.2
Alachlor	4	9	18	2	27	2.8	7.2
Alachlor	8	5	12	3	16	3.0	7.9
Alachlor	16	2	12	0	4	4.0	8.7
Metolachlor	1	11	44	14	61	2.8	6.3
Metolachlor	2	11	35	5	48	2.8	7.0
Metolachlor	4	6	24	2	31	2.8	8.2
Metolachlor	8	7	23	0	31	4.0	7.2
Metolachlor	16	4	15	0	19	6.0	6.9
Acetochlor	1	5	30	5	30	2.8	7.9
Acetochlor	2	5	13	1	20	3.0	8.2
Acetochlor	4	3	11	1	7	4.0	8.0
Acetochlor	8	3	4	0	4	5.3	7.9
Acetochlor	16	2	3	0	1	6.5	7.4
$LSD_{5\%}$		4	14	14	23	0.9	1.3

The maize grain yield was determined jointly by the weed control and the phytotoxic effects, which depended on the herbicide and the rate (Table 1). With a low rate of propachlor, the yield was lower due to the poor weed control effect, while the greatest yield was achieved at an extremely high rate (16 l ha^{-1}), due to the lack of phytotoxic effect. In the case of acetochlor, the opposite was observed: the yield was highest at a low rate due to the excellent weed control effect, while at a very high rate the phytotoxic effect inhibited a further increase in yield, or even led to a yield loss. The EWRC values (on a 1–9 scale) expressing the phytotoxic effect increased proportionately with the rate for all the herbicides.

2. Comparison of the phytotoxic effect of EPTC and butylate herbicides on maize as affected by temperature and antidote

The effects of various rates of the EPTC and butylate herbicides tested on plant height as affected by a continuous linear temperature gradient between 16°C and 26°C and by antidote are shown in Fig. 2. It can be seen that at 15.8°C the phytotoxic effect of herbicides was less evident, as the primary limiting factor was temperature. As the temperature increased from 15.8°C to 26.2°C, the phytotoxic effect of the herbicides differed more and more. Higher dosages led to increased phytotoxicity. In the case of EPTC the plant height – expressed as a percentage of the control – was between 30.0 and 38.2%. There was only a small increase in the damage from 16.7°C, and there was no significant difference between the phytotoxicity of higher rates. The phytotoxic effect of butylate was significantly less than that of EPTC (the shoot height as a percentage of control varied between 60.2 and 89.9, depending on dosage). The phytotoxic effect of different rates of butylate was highly dependent on temperature. There was a significant herbicide rate × temperature interaction. At higher rates of butylate the phytotoxic effect increased significantly above 20°C. The addition of antidote not only significantly reduced the phytotoxicity of EPTC and butylate in maize, but also reduced the influence of temperature on thiocarbamate injury.

3. Studies on the acetochlor and butylate sensitivity of maize hybrids and inbred lines

In a series of experiments, studies were made on the acetochlor sensitivity of 13 maize hybrids and 21 parental components (inbred lines, single crosses). A difference could be observed between the inbred lines as regards both their acetochlor sensitivity and the protective effect of the antidote AD-67. The shoot height of the lines most sensitive to acetochlor ranged from 16 to 32% of the control, averaged over the herbicide rates (1, 2 and 4 l ha^{-1}). In the case of the most tolerant lines the shoot height as a pecentage of the control varied between 60 and 77% when averaged over the herbicide rates. The protective effect of the antidote AD-67 was satisfactory, with a shoot height ranging from 72 to 86% as a percentage of the control and averaged over herbicide rates. Differences in acetochlor sensitivity were also observed for the hybrids (Berzsenyi et al., 1994). It can be concluded from the results that when the parents had different degrees of herbicide sensitivity, the acetochlor sensitivity of the hybrid was more likely to resemble the sensitivity of the maternal component, though exceptions to this rule were also found. The GR_{50} values (rate of acetochlor producing a 50% reduction in growth compared to the untreated control) of the most tolerant lines and hybrids (between 2 and 4.3 l ha^{-1}) showed a tolerance some three times greater than that of non-tolerant lines and hybrids (between 0.6 and 1.3 l ha^{-1}).

It was also found that the phytotoxic effect of acetochlor was well exhibited by both shoot height and plant mass, though the plant mass decreased to a greater extent, as illustrated clearly by the linear function fitted to the values of the two parameters ($Y = -4.40 + 0.948\ X$, $r = 0.968^{***}$, $n = 144$).

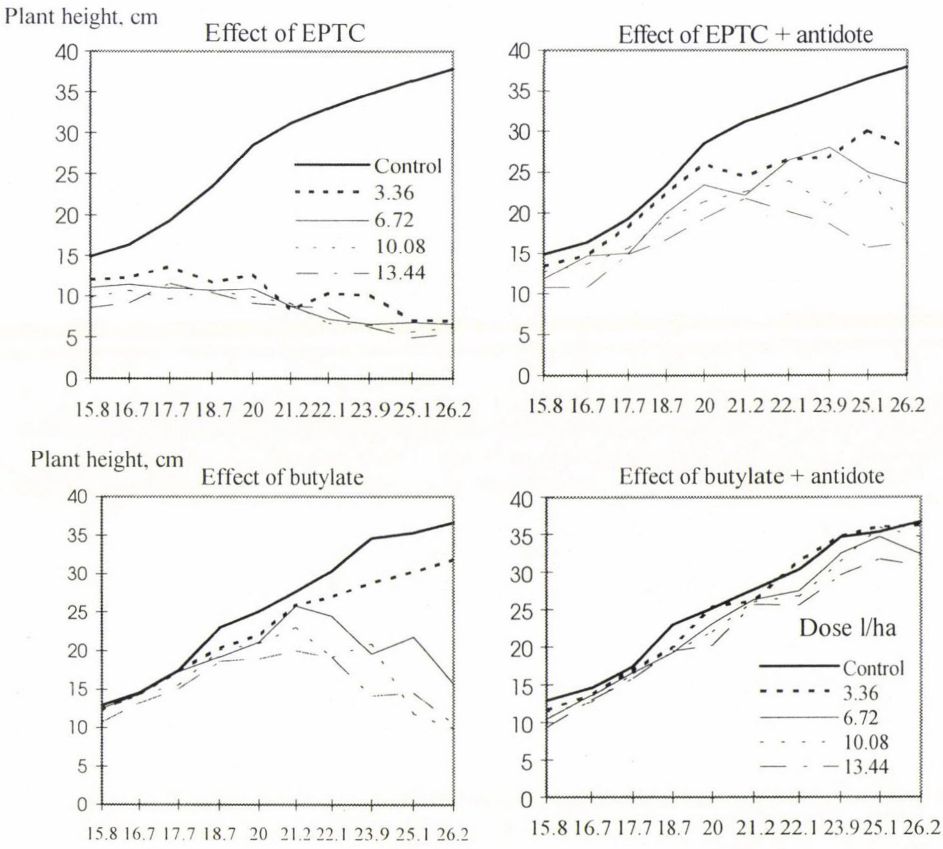

Fig. 2. Effect of temperature and antidote on the phytotoxic injury due to EPTC and butylate in gradient phytotron experiments

In a series of experiments, the butylate sensitivity of 46 parental components (inbred lines, single crosses) was tested. It was concluded that the order of magnitude of the damage was much smaller than that caused by acetochlor. In sensitive genotypes the shoot mass, as a % of the control, was 77–87% at a 5 l ha^{-1} rate of butylate, 62–76% at a 10 l ha^{-1} rate and 57–75% at a 15 l ha^{-1} rate. The antidote had a good protective effect, leading to a shoot green mass 74–98% of the control. For moderately sensitive parental components the reduction in shoot green mass was less intense, while the protective effect of the antidote led to green mass values of over 90%. It should be noted that the majority of parental components were not sensitive to butylate: even at the highest rate of butylate the shoot mass as a percentage of the control gave values of over 90%. In addition, no genotypes extremely sensitive to butylate were found among the 46 parental components examined.

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ZINC-MANGANESE RELATIONSHIPS IN BERSEEM (*TRIFOLIUM ALEXANDRINUM*) GROWN ON AN ALKALINE SOIL IN A POT EXPERIMENT

R. L. BANSAL and D. S. CHAHAL

DEPARTMENT OF SOILS, PUNJAB AGRICULTURAL UNIVERSITY, LUDHIANA-141004, INDIA

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The effect of Zn (0, 50, 100, 200 and 400 mg kg^{-1} soil from $ZnSO_4 \cdot 7H_2O$) and Mn (0, 50, 100 and 200 mg kg^{-1} soil from $MnSO_4 \cdot H_2O$) application on the dry matter yield and on the content and uptake of Zn and Mn by berseem grown on an alkaline soil was studied in a greenhouse experiment. The dry matter yield of berseem increased significantly with the application of 50 mg Mn kg^{-1}, but further addition had a non-significant effect on the yield. The addition of graded doses of Zn decreased the dry matter yield, which was 43.3% less than the control when 400 mg Zn kg^{-1} was applied. Zinc content and uptake increased with the application of either Zn or Mn. Manganese content and uptake also increased with the application of up to 200 mg Zn kg^{-1}, after which it decreased significantly. The significant reduction in dry matter yield and manganese uptake indicated the antagonistic effect of heavy applications of Zn on manganese utilization by berseem. The Zn-Mn disorder was significantly related to the Zn content as well as to the Zn/Mn concentration ratios in soil and plants. Zn/Mn concentration ratios of 27.3 in the soil and 11.0 in plant tissues were found to be critical, above which a reduction in the yield resulted.

Key words: Zn-Mn interaction, alkaline soil, berseem

Introduction

In India, in the early seventies, zinc was the most limiting factor in crop production and high Zn deficiency resulted in low yields even when NPK fertilizers were applied in sufficient quantities. Therefore, the consumption of zinc sulphate as a fertilizer, which was negligible in the early seventies, increased to more than 60,000 t in 1992. Due to the large scale use of zinc sulphate, Zn deficiency decreased in Punjab State from 90 to 30% (Arora et al., 1993). At the same time, the response obtained after zinc application to wheat, which originally varied from 0.8 to 1.7 t ha^{-1} (Takkar et al., 1973), decreased to 0.06 to 0.60 t ha^{-1} (Bansal et al., 1990). This appears to have resulted from the regular use of zinc fertilizers. Keeping in view the earlier high responses to zinc application, the farmers are now applying zinc sulphate even when it is not required. This may cause nutrient imbalances. There are reports that if the available Zn content in the soil, extracted with a 0.005M DTPA (diethylene triamine pentaacetic acid) buffer solution containing 0.1M triethanol amine and 0.01M $CaCl_2$ adjusted to pH 7.3 (Lindsay and Norvell, 1978), was above 45 mg kg^{-1} it resulted in severe Mn deficiency in wheat and significantly decreased the

Mn uptake by the crop despite more than adequate levels of Mn in the soil (Bansal et al., 1992). Therefore, the present investigation studied the effect of Zn and Mn application on growth and on the Zn and Mn contents and uptake in berseem (*Trifolium alexandrinum*) grown in an alkaline soil.

Materials and methods

A greenhouse experiment was conducted on a surface (0–15 cm) loamy sand soil belonging to the group Ustochrepts. The soil had pH (H_2O) 8.5, electrical conductivity 0.3 dSm^{-1} in 1:2 soil:water suspension at 25°C. The organic carbon and calcium carbonate contents were 0.48 and 0.70%, and the available P and K contents 8 and 115 mg kg^{-1} soil, respectively. The contents of organic carbon, calcium carbonate and available P in the soil samples were determined using the procedures described by Black (1965) and the available K by extraction with neutral normal ammonium acetate solution (Jackson, 1962). The available zinc and manganese contents extracted by the DTPA method of Lindsay and Norvell (1978) were 4.2 and 1.6 mg kg^{-1} soil, respectively. Polythene-lined earthen pots were filled with 4 kg soil. The treatments comprised five levels of zinc (0, 50, 100, 200 and 400 mg Zn kg^{-1} soil from $ZnSO_4.7H_2O$) and four levels of manganese (0, 50, 100 and 200 mg Mn kg^{-1} from $MnSO_4.H_2O$). All the pots received a basal application of 10 mg N and 13 mg P kg^{-1} soil in the form of urea and potassium dihydrogen orthophosphate, respectively. There were three pots for each treatment and the total number of pots in the experiment was 60. Eight seeds of berseem (cv. BL–1) were maintained in each pot. The representative soil samples were drawn from each pot after harvesting the crop.

The plant samples were washed with 0.1N HCl, distilled and deionized water, dried at 70°C and ground in a stainless steel mill to pass through a 20 mesh sieve. The plant material was wet ashed on a nitric-perchloric-sulphuric acid mixture. The soil samples were analysed for Zn and Mn by extraction with 0.005M DTPA (diethylene triamine pentaacetic acid) buffer solution containing 0.1M triethanol amine and 0.01M $CaCl_2$ adjusted to pH 7.3 with distilled HCl, using a soil solution ratio of 1:2 and a shaking time of 2 hours (Lindsay and Norvell, 1978). The Zn and Mn contents in soil and plant extracts were determined by atomic absorption spectrophotometry.

Results and discussion

Dry matter yields

In the absence of applied Zn, the dry matter yield of berseem increased significantly with the application of 50 and 100 mg Mn kg^{-1} soil, but decreased significantly with the application of 200 mg Mn kg^{-1} soil compared with the 50 mg Mn kg^{-1} soil treatment and remained at par with the no Mn soil treatment (Table 1). The increase in yield with Mn application was mainly due to the deficiency of Mn in the soil (DTPA extractable Mn 1.6 mg kg^{-1}). As the soil was already high in available Zn, the addition of graded doses of Zn decreased the dry matter yield of berseem and the decrease was significant at and above 200 mg Zn kg^{-1} soil application. At 400 mg Zn kg^{-1} soil, the dry matter yield declined from 15.0 g pot^{-1} in the control to 8.5 g pot^{-1} . The data indicated that a combined application of 50 mg Mn and 0 mg Zn kg^{-1} soil produced the maximum dry matter. The application of either of these nutrients beyond these levels had a negative effect on the dry matter yield. This depressing effect was,

Table 1

Effect of Zn and Mn fertilization on the dry matter yield and on Zn and Mn contents in plants

Treatment (mg kg^{-1} soil)		Dry matter yield (g pot^{-1})	Nutrient conc in plants (μg g^{-1})		Nutrient uptake in plants (mg pot^{-1})		Soil-available nutrient (mg kg^{-1})	
Zn	Mn		Zn	Mn	Zn	Mn	Zn	Mn
0	0	14.2	77	21	1.09	0.30	4	1.6
50	0	13.9	210	25	2.92	0.35	32	1.5
100	0	13.5	396	28	5.34	0.38	60	1.4
200	0	11.8	721	36	8.51	0.42	120	1.4
400	0	9.0	1284	34	11.56	0.31	280	1.3
0	50	15.8	108	36	1.70	0.57	4	5.2
50	50	14.9	216	52	3.22	0.77	35	6.0
100	50	14.2	399	67	5.66	0.95	65	5.9
200	50	12.3	783	86	9.63	1.06	125	5.7
400	50	9.5	1540	58	14.63	0.55	305	5.2
0	100	15.6	118	56	1.84	0.87	4	9.2
50	100	15.1	223	64	3.37	0.97	38	12.0
100	100	14.5	421	83	6.10	1.20	50	11.0
200	100	12.1	844	126	10.21	1.52	127	10.2
400	100	8.4	1718	81	14.43	0.68	310	9.0
0	200	14.3	134	61	1.92	0.87	4	15.7
50	200	15.4	261	179	4.02	2.76	36	14.0
100	200	15.2	551	205	8.37	3.11	55	12.5
200	200	11.8	1099	234	12.97	2.76	122	11.0
400	200	7.0	2040	158	14.28	1.10	312	9.5
LSD at P = 0.05								
Zn		0.6	77	12	0.8	0.17	10	NS
Mn		0.7	87	14	0.9	0.19	NS	2.0
Zn+Mn		1.4	174	28	1.8	0.38	NS	NS

NS = non–significant

however, most prominent at the highest level of application and more so in the case of Zn. The decrease in dry matter yield varied from 1.3 to 43.0% with the increase in Zn levels from 50 to 400 mg Zn kg^{-1}, whereas it was only between 1.5 to 4.5% with the application of 100 and 200 mg Mn kg^{-1} compared with 50 mg Mn kg^{-1}. The highest yield, achieved with Zn_0Mn_{50}, resulted from the favourable influence of both the nutrients essential for plant growth, as the soil-available Zn and Mn values at this level were 4.4 and 5.2 mg kg^{-1} and both the values are above the critical deficiency levels. Therefore, the subsequent decrease in dry matter yield in the presence of high doses of either of the two nutrients may be ascribed to ionic imbalances. Similar results have been reported for maize by Singh and Steenberg (1974).

Zinc content and uptake

The zinc content in the plants increased significantly with the increase in Zn application. The mean Zn content was 109, 227, 442, 861 and 1645 $\mu g\ g^{-1}$ at 0, 50, 100, 200 and 400 mg Zn kg^{-1} soil, respectively. The addition of Mn up to 50 mg kg^{-1} did not have a great influence on the Zn content, but higher rates of 100 and 200 mg Mn kg^{-1}, combined with 200 and 400 mg Zn kg^{-1} resulted in a significant increase in the Zn content. The plant Zn content varied from 77 $\mu g\ g^{-1}$ in the control to 1284 $\mu g\ g^{-1}$ after the addition of 400 mg Zn kg^{-1}. There appears to be a positive relationship between soil Zn additions and the translocation of Zn in the plant.

The average Zn uptake by the plant significantly increased with the rate of Zn application. Manganese application also significantly increased the Zn uptake. The increase in Zn uptake was of the order of 1.74, 4.73, 8.69 and 12.08 mg pot^{-1} at 50, 100, 200 and 400 mg Zn kg^{-1} soil, respectively as compared to no–Zn treatment. The addition of Mn increased the mean Zn uptake over the control by 18.5, 22.2 and 41.3%, respectively, at 50, 100 and 200 mg Mn kg^{-1}, indicating that the translocation of Zn to the plant is affected by Mn application. Reddy and Dunn (1987) reported that the Zn content in soybean was not influenced by Mn application. Singh and Steenberg (1974) also found that the uptake of Zn in maize and barley was not affected by Mn application except at the highest concentration of Zn, where a marginal effect was noticed. The present results, however, indicated that the application of Mn resulted in increased Zn content and uptake in berseem.

Mn content and uptake

There was a significant increase in the plant Mn content with an increase in the rate of Mn application at all levels of applied Zn. Increasing rates of Zn application tended to increase the Mn content except at 400 mg Zn kg^{-1} where it significantly decreased the Mn content in the plant.

The average Mn uptake by the plants was significantly increased compared with the control after Mn application. Irrespective of the Mn treatment the mean uptake of Mn increased in the presence of Zn though it decreased significantly at 400 mg Zn kg^{-1}. The results indicated that the application of 200 mg Mn and 100 mg Zn kg^{-1} resulted in the maximum uptake of Mn. The application of 400 mg Zn kg^{-1} markedly reduced the mean Mn uptake. The decrease in Mn uptake with increased Zn application may be ascribed to the decrease in dry matter yield at higher levels of applied nutrients and to ionic competition between Mn and Zn in the process of absorption.

Singh and Steenberg (1974) reported that Mn absorption by maize and barley plants decreased markedly with an increase in the rate of Zn application to the soil. Ishizuka and Ando (1968), working with rice plants, found that the amount of Mn absorbed by the plants was markedly reduced at increasing Zn concentrations. A similar antagonistic effect between Zn and Mn was observed in corn (Hulagur and Dangarwala, 1982) and soybean (White et al., 1979).

Prediction parameters of Zn-Mn disorder in plants

Values of 250 mg kg^{-1} zinc in the soil (Brune and Ellinghans, 1981) and 702 μg g^{-1} Zn in alfalfa plants (MacLean, 1974) have been reported as the toxicity levels above which a significant reduction in yield occurs. In the present investigation, the soil Zn ranged from 4.2 to 312.0 mg kg^{-1} and the plant Zn from 77 to 2040 μg g^{-1} (Table 1), indicating a toxic concentration of Zn in the soil and plants studied. There was a significant but negative coefficient of correlation between the dry matter yield and the Zn content of the soil ($r = -0.960$**) and the plants ($r = -0.956$**) (Table 2). However, the Mn concentrations in the soil and plants were non-significantly correlated with the dry matter yield. Therefore, an attempt was made to determine the coefficient of correlation between dry matter yield and the Zn/Mn concentration ratios in soil and plants. There was a significant correlation between dry matter yield and the Zn/Mn concentration ratios in the soil ($r = -0.577$**) and the plants ($r = -0.766$**). This suggests that Zn-Mn disorders in plants should be monitored using Zn/Mn concentration ratios rather than their concentrations alone. The statistical model of Cate and Nelson (1971) was followed to determine the critical toxicity levels. These were 203 mg kg^{-1} Zn in soil and 1190 μg g^{-1} Zn in plants. Zn/Mn concentration ratios of 27.3 in the soil and 11.0 in plants were found to be the critical values above which a reduction in the yield occurs.

Table 2
Coefficients of correlation between dry matter yield and nutrient content in soil and plants

Parameter	Coefficient of correlation (r)
In soil	
Zn	–0.960**
Mn	0.197 NS
Zn/Mn ratio	–0.577**
In plants	
Zn	–0.956**
Mn	–0.421 NS
Zn/Mn ratio	–0.766**

** Significant at 1% level. NS = Non-significant

The present study suggested that Zn application up to 200 mg kg^{-1} enhances the Mn availability in plants, whereas at higher rates, i.e. 400 mg Zn kg^{-1}, it has an antagonistic effect on Mn availability to the plants. Mn application has a positive and synergistic effect on the Zn availability in berseem.

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DATA ON THE APPLICATION OF ADIPOCYTE MORPHOMETRY IN YOUNG HOLSTEIN BULLS IN HUNGARY

J. TŐZSÉR[1], A. HIDAS[2], M. MÉZES[3], J. AGABRIEL[4], A. KOVÁCS[1], E. SZŰCS[1] and I. HOLLÓ[5]

[1]INSTITUTE OF ANIMAL HUSBANDRY, FACULTY OF AGRICULTURE, UNIVERSITY OF AGRICULTURAL SCIENCES, GÖDÖLLŐ, H - 2103, HUNGARY
[2]RESEARCH CENTRE FOR SMALL ANIMAL BREEDING AND NUTRITION, GÖDÖLLŐ, H - 2103, HUNGARY
[3]DEPARTMENT OF NUTRITION, FACULTY OF AGRICULTURE, UNIVERSITY OF AGRICULTURAL SCIENCES,GÖDÖLLŐ, H - 2103, HUNGARY
[4]RESEARCH CENTRE, CLERMONT-FERRAND-THEIX, 63122, FRANCE
[5]FACULTY OF ANIMAL SCIENCE, PANNON AGRICULTURAL UNIVERSITY, KAPOSVÁR, H–7401, HUNGARY

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In Hungary adipocyte morphometry based on the size of the fat cells *in vivo* has not previously been used. Thirty-one young fattening bulls of the Holstein breed were studied at a feed-lot farm in Zirc, Hungary. The feeding of the young bulls tested was based on maize silage, meadow hay *(ad libitum)* and concentrate (limited) during a fattening period of 253 days. Three subcutaneous adipose tissue samples (I, II, III) were taken during the fattening period from the rump. Adipose cells were fixed by osmium tetroxide and isolated in urea solution as proposed by Robelin and Agabriel (1986). The fixed cells were trapped on a cellulose nitrate filter (Sartorius). The adipocyte diameter was measured by image analysis (Cytosoft®). The bulls were sent to slaughter at the age of 609 days on average. The gut and its water content were also determined at slaughter. Then, after 24 hours of chilling, the lefthand half carcasses were dissected. A significant growth (+18.6μ, $P<0.001$) in the adipocyte diameter was observed. The adipose cell diameter combined with live weight gave a reasonably good estimation of total body fat content ($R^2=0.61$, $P<0.001$). The percentage coefficient of determination (R^2) from the live weight, empty body weight and adipocyte diameter in the estimation of the lean-to-fat ratio was less than 50% ($R^2=46$ and 39). The use of adipocyte morphometry by Hungarian breeders was recommended by the authors.

Key words: fat cell diameter, adipocyte morphometry, lipid deposition, estimating body lipids, young bull

Introduction

One of the main goals in meat production research is to decrease lipid deposition and to increase protein deposition. The deposition of fat and adipose tissue cellularity are influenced by genetics, breed, sex, growth, diet, hormones and other environmental factors. Studies on the cellularity of adipose tissue have been reported for humans (Hirsch and Knittle, 1970; Salan et al., 1971), rats (Hirsch and Han, 1969; Johnson et al., 1971), mice (Johnson and Hirsh, 1972) and swine (Anderson and Kauffman, 1973). Bell (1909), Robelin (1985) and Wegner and Matthes (1994) reported on the increase in the fat cell diameter of bovine muscle during growth. Moody and Cassens (1968), Robelin (1981), Schiavetta et al. (1990) and Robelin and Casteilla (1990) found that bovine adipose cells varied in size according to depot site (subcutaneous, intermuscular, interfascicular).

A good prediction of the body composition of the living animal is essential for the determination of fat or energy efficiency. Several methods varying in cost and accuracy have been investigated for cattle: body condition scoring (Agabriel et al., 1986), ultrasonic scanning of fat depth (Miles et al., 1983), measuring the speed of ultrasound (Miles et al., 1984), measurement of fat cell sizes (Robelin and Agabriel, 1986) and a technique involving the dilution space of deuteriated water (Cowan et al., 1979; Robelin, 1982).

Renand et al. (1992) demonstrated on 79 young Charolais bulls that the best *in vivo* total body fat content prediction was obtained by adipose cell size ($R^2 = 0.63$), followed by the live velocity of ultrasound ($R^2 = 0.59$) and fat depth scanning ($R^2 = 0.51$). According to Renand et al. (1996) the highly positive correlation between adipose cell diameter and fat content ($r = 0.56$) indicates that this characteristic could also be used for the further improvement of selection.

Several prediction equations of the body lipids are proposed by Robelin and Agabriel (1986) and Robelin et al. (1989) for use by animal breeders (Table 1).

Table 1

Estimation of lipids from body weight, deuteriated water space and adipose cell diameter by breeds and sex

Breeds	Sex	Prediction equations
Holstein	Cow	Lip= 0.905*BW – 1.140*DWS Lip= –160.8 + 0.272*BW + 0.964*DIAM Lip= 0.144*DIAM + 3.88
	Bull	Lip = 0.068*DIAM +7.46
Charolais	Cow	Lip= 0.798*BW – 0.975*DWS Lip= –174.6 + 0.315*BW + 0.879*DIAM Lip= 0.144*DIAM – 0.12
	Bull	Lip= 0.068*DIAM + 6.33
Limousine	Cow	Lip= 0.815*BW – 1.407*DWS Lip= –172.1 + 0.325*BW + 0.736*DIAM Lip= 0.144*DIAM + 0.61
	Bull	Lip= 0.068*DIAM + 4.24

Legend: Lip=lipids, kg; BW=body weight, kg; DWS=deuteriated water space, kg; DIAM=adipose cell diameter, microns

In Hungary adipocyte morphometry concerning the size of the fat cells *in vivo* has not previously been used. The objective of the investigation was to demonstrate the evolution of adipose cell size during the fattening period in Holstein-Friesian young bulls. In addition, prediction equations were calculated to estimate the body lipids and the lean-to-fat ratio from body weight, empty body weight and adipose cell diameter.

Materials and methods

Thirty-one young Holstein-Friesian fattening bulls were used in this experiment at a feed-lot farm in Zirc, Hungary. The animals were fed diets based on maize silage, meadow hay (*ad libitum*) and concentrate (limited) during a fattening period lasting for 253 days. Three subcutaneous adipose tissue samples (I, II, III) were taken during different phases of the fattening period from the rump region. Adipose cells were fixed by osmium tetroxide and isolated in urea solution as proposed by Robelin and Agabriel (1986) (Table 2). The fixed cells were trapped on a cellulose-nitrate filter (Sartorius). The adipocyte diameter was measured by image analysis (Cytosoft® software). Bulls were sent to slaughter at the age of 609 days on average. The gut and its water content were also determined at slaughter. Then, after 24 hours of chilling, the left hand half carcasses were dissected.

Prediction equations for total body fat content and lean-to-fat ratio were calculated by multiple regression analysis.

Table 2

Details of the adipocyte morphometry method

Preparation	Solutions: – Tyrode – Sörensen A (1.56 g/1000 ml) and B (1.45 g/1000 ml) – Osmium tetroxide (1 g/25 ml) – NaCl (9 g/1000 ml)
Execution	– biopsy sample of rump adipose tissue – cutting, washing, filtering (filter apparatus manufactured by Sartorius, cellulose nitrate filter, pore size, 0.45) – fixation and incubation of samples (for 72 – 96 hours, at 39°C)
Evaluation	200 cells per tissue sample measured by image analysis (Cytosoft®)

Results and discussion

The data of the traits analysed are summarised in Table 3. The average final fattening weight agreed with the results of Robelin (1985) and Szabó et al. (1993a). This tendency was also confirmed by the data obtained from the dissection of the lefthand half carcasses. The fat percentage (6.4%, trimmed fat kg/carcass kg *100) in this study was significantly smaller than that (11.3%) observed by Szabó et al. (1993b). These results can be explained by the low average daily weight gain (714 g/day) during the fattening period.

Table 3
Measurement data of young Holstein-Friesian fattening bulls (n=31)

Measurements	I	II	III
Age, days	356.0±26.34	446.0±26.34	608.0±26.99
Live weight, kg	346.3±31.80	404.7±43.13	526.9±49.99
Adipocyte diameter of the rump adipose tissue, μ	84.9^a±9.67	87.4^a±11.03	103.5^a±9.72
Weight of carcass halves, kg			311.2±32.76
Dressing %			59.0±1.51
Lefthand half carcass (cold), kg			142.4±12.57
Total body fat content from trimmed fat, paunch fat, intestinal fat, kg			22.2±6.62
Lean-to-bone ratio			3.6±0.23
Lean-to-fat ratio			10.3±3.0

Means on the same line with different superscripts differ significantly at $P<0.001$

Significant growth was observed (+18.6μ, $P<0.001$) in the adipocyte diameter of rump adipose tissue. The adipocyte diameters measured for Holstein bulls (84.9; 87.4; 103.5 μ) were similar to findings published by Hood and Allen (1973), Robelin (1981) and Wegner et al. (1993). The distribution of the cells according to adipocyte diameter is presented in Figures 1–3.

The distribution of the adipocyte diameter at the start of the experiment shows clearly that the most frequent adipocyte diameter (45.2%) was 80–90 μ (Fig. 1). A similar tendency (41.9%) was found in the course of the experiment (Fig. 2), though the proportion of cells in the 5th category (diameter: 100–110 μ) (12.9%) was approximately twice as high as in Figure 1. The distribution of adipocyte diameters at the end of the experiment (shown in Fig. 3) reveals an increase in the frequency of the 3rd, 4th and 5th categories (38.7; 29; 29%). This phenomenon was previously described by Hood and Allen (1973) and Robelin (1985). The absence of small cells (less than 70 μ) in the Holstein subcutaneous tissues indicated that cellular hyperplasia was complete in the young bulls after 13 months of life.

Table 4 gives the results of multiple regression analysis. The adipose cell diameter combined with live weight gave a somewhat better estimation of total body fat content ($R^2 = 0.61$, $P<0.001$) than the other combination of estimation ($R^2 = 0.58$, $P<0.001$, empty body weight and adipocyte diameter). The estimation of total body fat content from adipocyte diameter and live weight was very close to the results of Renand et al. (1992) ($R^2 = 0.63$).

The percentage coefficient of determination (R^2%) for live weight, empty body weight and adipocyte diameter in the estimation of the lean-to-fat ratio is given in Table 5. In both cases the total accuracy was less than 50%

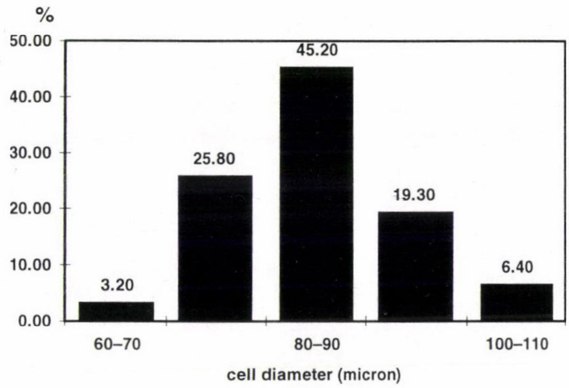

Fig. 1. Size distribution of adipose tissue cells at the start of the experiment

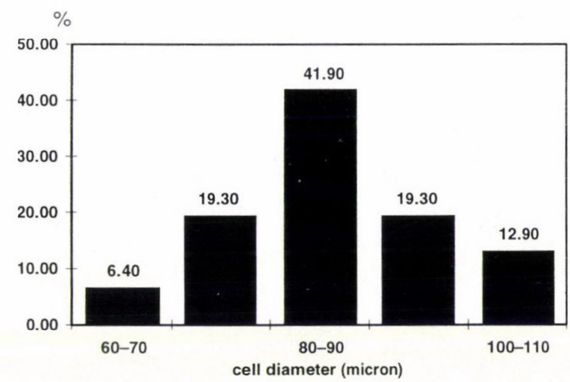

Fig. 2. Size distribution of adipose tissue cells during the experiment

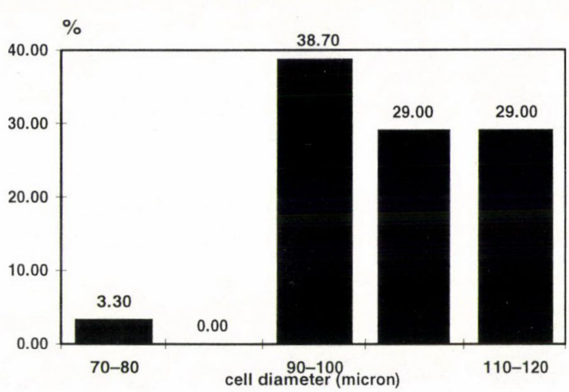

Fig. 3. Size distribution of adipose tissue cells at the end of the experiment

Table 4
Prediction equations for total body fat content from live weight, empty body weight and adipocyte diameter in young Holstein bulls

Independent variables (x)	Dependent variable (y)	Regression equations ($y = bx_1 + bx_2 + c$)	Multiple correlation coefficients (R)	Determination coefficients (R^2 %)
Live weight, kg, x_1 Adipocyte diameter of the rump adipose tissue, μ, x_2	Total body fat content, kg	$y = 0.1035x_1 - 0.0215x_2 - 30.10$	0.78***	61
Empty body weight, kg, x_1 Adipocyte diameter of the rump adipose tissue, μ, x_2	Total body fat content, kg	$y = 0.1191x_1 - 0.0619x_2 - 24.67$	0.76***	58

***=P<0.001, n=31

(R^2% = 46 and 39). However, Renand et al. (1992) found that the accuracy of the estimation of carcass lean content from the live weight, fatness score, fat depth scanning, velocity of ultrasound and adipose cell diameter varied to a large extent (R^2% = 1.0–47). In a study carried out by Renand et al. (1992) the estimation of carcass lean content from the adipocyte diameter had an accuracy of less than 30%.

Table 5
Prediction equations for lean-to-fat ratio from live weight, empty body weight and adipocyte diameter in young Holstein bulls

Independent variables (x)	Dependent variable (y)	Regression equations ($y = bx_1 + bx_2 + c$)	Multiple correlation coefficients (R)	Determination coefficients (R^2%)
Live weight, kg, x_1 Adipocyte diameter of the rump adipose tissue, μ, x_2	Lean-to-fat ratio	$y = -0.0400x_1 - 0.057x_2 - 37.38$	0.68***	46
Empty body weight, kg, x_1 Adipocyte diameter of the rump adipose tissue, μ, x_2	Lean-to-fat ratio	$y = -0.0444x_1 - 0.0418x_2 + 34.41$	0.63***	39

***=P<0.001, n=31

Based on the results of this study, the use of adipocyte morphometry can be recommended to Hungarian breeders, because a good estimation of the *in vivo* total body fat content was provided by adipose cell size ($R^2 = 0.61$).

These first investigations need to be repeated and confirmed with new samples of cattle from diverse breeds.

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Short communication

WHOLE LEAF THERMOLUMINESCENCE AS A PROSPECTIVE TOOL FOR MONITORING INTRASPECIFIC COLD TOLERANCE IN CROP SPECIES

J.-M. DUCRUET*, T. JANDA and E. PÁLDI

*SBE, BAT 532, INRA/CEA SACLAY, 91191 GIF-SUR-YVETTE, CEDEX, FRANCE
AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES, H–2462 MARTONVÁSÁR, P.O.BOX 19, HUNGARY

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In maize or pea plants cultivated at 17/14°C, leaf thermoluminescence measurements showed both a decrease in the activation energy of the B-band induced by one flash and a temperature downshift of the AG-band induced by far-red light, observed only in cold- or chilling-tolerant plants as compared to unhardened or sensitive plants. This suggests the existence of cold-tolerance mechanisms within the photosynthetic membranes and may lead to a rapid test for screening tolerant genotypes.

Key words: maize, *Zea mays*, pea, *Pisum sativum*, photosynthesis, chilling stress, thermoluminescence

Introduction

The photosynthetic apparatus is an important target of chilling/cold stress, since light energy absorbed in excess induces phototoxic damage in the cell. More specifically, photoinhibition induced by high light at low temperature corresponds to a breakdown of photosystem II (PS-II).

The preliminary results presented here demonstrate how the thermoluminescence of intact leaves might be used to investigate the effects of chilling temperatures on photosynthetic membranes, with the prospect of assaying the chilling resistance of crop plant genotypes.

Materials and methods

Pea varieties and maize inbred lines were grown in PGR climatic chambers in the Martonvásár phytotron (Tischner et al., 1997) and were progressively cold-hardened.

Thermoluminescence (TL) results from the recombination of charge pairs previously separated, due to illumination. After light excitation of a leaf fragment at 0°C or below, TL is subsequently detected in the dark, during slow heating. A transportable TL apparatus built in Saclay (Miranda and Ducruet, 1995) has been installed in Martonvásár.

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Results and discussion

Two types of TL emission provide information about PS-II modifications due to hardening:

Variation of the activation energy E_a of the B-band

This constitutes the most simple situation, in which a single turn-over flash (5 μs), given at 0°C or below, separates a +/– charge pair. This pair recombines during progressive warming, producing, at low yield a B-band peaking near 30°C. An apparent energy E_a of recombination can be derived from the analysis of TL curves. This corresponds to the energetic barrier which makes photosynthesis possible by stabilising the light-induced +/– charge pairs, thus limiting energetic loss through the back-reaction of charge recombination.

Using 3 cold-tolerant and 2 cold-sensitive pea cultivars, similar E_a values of the B-band were found in unhardened plants grown above 20°C (1.0 to 1.1 eV), whereas E_a only decreased (Fig. 1) upon cold-hardening in the tolerant cultivars (down to 0.8 eV at 5°C).

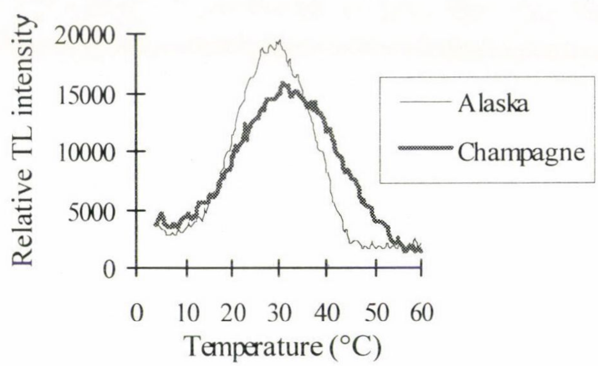

Fig. 1. The B-band in Alaska (cold-sensitive) and Champagne (cold-tolerant) pea varieties induced by 1 flash at 1°C

This decrease in E_a was observed after a light period at a hardening temperature and was slowly reversed in the dark. A similar decrease was observed in some – but not all – tolerant maize lines cultivated at 18/14°C (not shown). This decrease in the E_a activation energy is generally observed in cold-tolerant plants such as spinach (Briantais et al., 1992).

A downshift of the AG-band induced by far-red light

The AG or afterglow band peaking near 45°C is induced by far-red (PS-I exciting) light and originates from PS-II, but reflects a complex phenomenon of reverse electron flow involving the whole photosynthetic electron transport chain (Miranda and Ducruet, 1995). A downshift of this AG-band was repeatedly observed in several chilling-tolerant maize plants hardened below 20°C (18/14°C) in the light, then dark-incubated from 1 to 20 h, whereas this downshift was lower in unhardened or hardened chilling-sensitive lines (Fig. 2). It progressively faded out in the dark and, beyond 24 hours, the usual location near 45°C of the AG-band was restored in all lines. Such a downshift has also been observed in other chilling-tolerant maize lines (F2 compared to the sensitive line MBS 847) and in grapevine cultivars (unpublished results).

The two above-reported effects of hardening on the B and AG bands of TL in tolerant genotypes can both be tentatively ascribed to a change in the lipid environment around PS-II (increased fatty acid unsaturation) and/or to a phosphorylation of proteins of the PS-II complex. This agrees with the results of Bergantino et al. (1995) who showed that a chilling-tolerant maize, unlike a sensitive one, was able to phosphorylate a CP-29 subunit of the PS-II antenna. This would prevent an excess of absorbed energy from reaching the PS-II centres at chilling or cold temperatures. However, the hardening effects reported here are detected to various extents in tolerant lines, suggesting that different mechanisms contribute additively to tolerance, one of which can be evidenced by TL measurements.

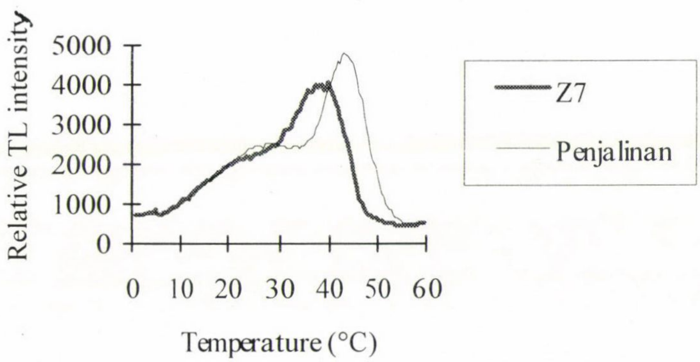

Fig. 2. The AG-band in Z7 (cold-tolerant) and Penjalinan (cold-sensitive) inbred maize lines measured after 30 s far-red illumination at 0 °C

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Book review

WILLIAM S. KLUNG and MICHAEL R. CUMMINGS: *Concept of Genetics.* Fifth Edition. Prentice Hall International, Inc. Simon and Schuster/A Viacom Company, Upper Saddle River, New Yersey 017458. ISBN 013–724410–X

I am familiar with many textbooks on genetics, and have written three myself, but I have never yet met with such a warmly human dedication. This book is dedicated to students of the authors, who contributed to the success of the book with their enthusiasm for the subject and with their inquisitive interest.

The authors have made an excellent job of their task of teaching students how to think in terms of genetics as regards both the subject matter, methods, illustrations and data presentation.

Apart from the Introduction, the book is divided into three main sections: Heredity and the Phenotype, with 9 chapters, Molecular Basis of Heredity, with 8 chapters, and Advanced Topics in Genetic Analysis, with 8 chapters. Compared to previous editions, the chapters which have been revised to the greatest extent are those on the Application of Recombinant DNA Technology, Genetics and Cancer, the Genetic Basis of the Immune Response, Population Genetics, and Genetics and Evolution.

I should like to make special mention of the passages entitled "Genetics, Technology and Society". The authors are of the opinion that the development of genetics is linked to social questions and technological solutions, and that these will have an enormous effect on human destiny in the next century.

The structure of each chapter is excellent from the didactic point of view. After a brief introduction of the subject there follows a well-illustrated exposition, with a strictly historical approach, providing a clear picture of scientific development right up to the present. After a brief summary a section entitled Insight and Solution puts the knowledge gained into practice through the solution of various examples. A similar purpose is served by the section Problems and Discussion Questions. The Extra-Spice Problems are unique. Probably due to the lecturing background of the authors, a considerable portion of the examples are taken from the field of human genetics, but in each chapter all the topics are discussed in detail irrespective of the object on which the research was carried out.

This review cannot deal in detail with all the chapters of the book. Personally, I found the greatest pleasure in the chapter on evolution. The evolution of the living world is not treated as a separate subject in agricultural universities, but in my own textbooks evolution is included in the title and makes up approx. 1/3 of the book. Together with genetics, this forms the background for the teaching of plant breeding: according to Vavilov (1950) plant breeding is simply evolution controlled by man. The book gives an excellent summarisation of the results achieved in both traditional and molecular research on evolution.

Among the appendices, the excellent Glossary is worth special mention. It is the general opinion that biological sciences are developing at such a rate that the sum of knowledge is doubled every 10 years. The authors consider this rate to be every 5 years for genetics. It is rarely possible to publish a new textbook this often. From this point of view, too, the authors have done excellent work and have every right to expect the appreciation and interest of a wide range of people interested in genetics.

A. BÁLINT

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